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THE EFFECT OF ANTI-GLYCOPHORIN A MONOCLONAL ANTIBODIES AND FAB FRAGMENTS ON PLASMODIUM FALCIPARUM ERYTHROCYTE INVASION IN VITRO

Stephen Joel Roth

1986

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THE EFFECT OF ANTI-GLYCOPHORIN A MONOCLONAL ANTIBODIES AND FAB FRAGMENTS ON *PLASMODIUM FALCIPARUM* ERYTHROCYTE INVASION *IN VITRO*

A Thesis Submitted to the Yale University
School of Medicine in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Medicine

Stephen Joel Roth

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ABSTRACT

THE EFFECT OF ANTI-GLYCOPHORIN A MONOCLONAL ANTIBODIES AND FAB FRAGMENTS ON *PLASMODIUM FALCIPARUM* ERYTHROCYTE INVASION *IN VITRO*

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Merozoites of the malaria parasite *Plasmodium falciparum* recognize and attach to erythrocyte sialoglycoproteins when invading human erythrocytes. On the major sialoglycoprotein, Glycophorin A (GpA), attachments appear to occur both to oligosaccharides on the NH_2-terminal portion of the molecule and to a site close to the erythrocyte membrane. Three anti-GpA monoclonal antibodies, R7, R10, and R18, and their Fab fragments were tested for the ability to inhibit *P. falciparum* erythrocyte invasion using *in vitro* invasion assays. Only R7, which is directed against a region of GpA near the membrane, produced strong inhibition both as an intact antibody and in Fab fragment form. GpA cross-linking and erythrocyte agglutination, two potential inhibitory mechanisms complicating the interpretation of the results with intact antibody, are ruled out by this result with R7 Fab fragments. The pattern and degree of inhibition caused by R10 and R18, which are directed against sites on GpA further from the membrane, suggest that they inhibit by a mechanism such as steric hindrance. The results of these studies provide additional evidence that a site on GpA close to the

erythrocyte membrane is critical for *P. falciparum* invasion. Preliminary work with BRC14, an anti-GpA monoclonal antibody with the same serological specificity as R7, gave results similar to those observed with R7.

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INTRODUCTION

Malaria is a parasitic disease of both humans and a number of animal species which is caused by protozoa of the genus *Plasmodium*. Human malaria, targeted for global eradication by the World Health Organization as recently as the 1960s, has increased two- to three-fold in prevalence worldwide during the past 10-15 years (1). The reasons for this resurgence are complex and include a variety of biological, cultural, and economic factors (2). Of the four species of human malaria parasites, *Plasmodium falciparum* causes the majority of the morbidity and mortality associated with malarial disease. Because of this notorious preeminence and recent advances in *in vitro* culturing techniques, efforts to develop new anti-malarial pharmacologic and immunologic agents have been focused primarily on *P. falciparum*.

Erythrocyte invasion by *P. falciparum* is a complex, multi-step event which initiates the pathophysiologic processes that lead to disease in man. The invasive form of the parasite, the merozoite, must recognize and attach to an erythrocyte, orient its apical end towards the cell's surface, form a movable junction with the cell's membrane, and finally enter the cell by a specialized type of endocytosis without destroying it.

Although the detailed mechanisms of erythrocyte invasion are still unknown, it appears that *P. falciparum*

merozoites recognize and attach to specific erythrocyte receptor molecules. These receptor molecules are the human erythrocyte sialoglycoproteins or glycophorins. Because erythrocyte invasion is critical for parasite survival in man, a better understanding of the merozoite-glycophorin interaction may lead to the development of new and effective methods for blocking merozoite invasion. This appraoch would complement current efforts to develop vaccines against the sporozoite form of the parasite.

The first three sections of this thesis describe the P. falciparum life cycle, the details of merozoite invasion as observed through the light and electron microscope, and the experimental data which support the role of glycophorins as receptor molecules for P. falciparum merozoites. The fourth and fifth sections provide the materials, methods, and results of a series of experiments performed to assess the ability of anti-glycophorin A monoclonal antibodies and their Fab fragments to inhibit P. falciparum invasion. In the final section, these experimental results are discussed and interpreted in the context of our current understanding of the erythrocyte invasion process.

THE PLASMODIUM FALCIPARUM LIFE CYCLE

The P. falciparum life cycle (3) consists of separate sexual and asexual cycles in two different host organisms, the Anopheles mosquito and man (Figure 1). The asexual cycle in man is initiated when an infected female Anopheles mosquito injects saliva harboring sporozoites into a human while attempting to obtain the blood needed to produce her eggs. Inside the human host, sporozoites attach to and then invade hepatocytes. Each sporozoite develops first into a form of the parasite called a trophozoite and later into a schizont. The schizont consists of thousands of nuclear masses (up to 30,000/hepatocyte) which derive from the mature trophozoite nucleus by asexual division. Each nuclear mass becomes a daughter parasite called a merozoite. The entire proliferative process lasts approximately one week, at which time the mature schizont within a hepatocyte ruptures and liberates its merozoites.

As merozoites enter the bloodstream from the liver parenchyma, they rapidly attach to and invade erythrocytes. Invasion starts an intra-erythrocytic cycle of development in which the merozoite grows into a young trophozoite called a ring form and then progresses through the trophozoite stage until the schizont stage is reached again. In the erythrocyte only between 8 and 32 merozoites (the average number being 16) are produced per schizont. When the schizont is mature, it ruptures and releases its merozoites

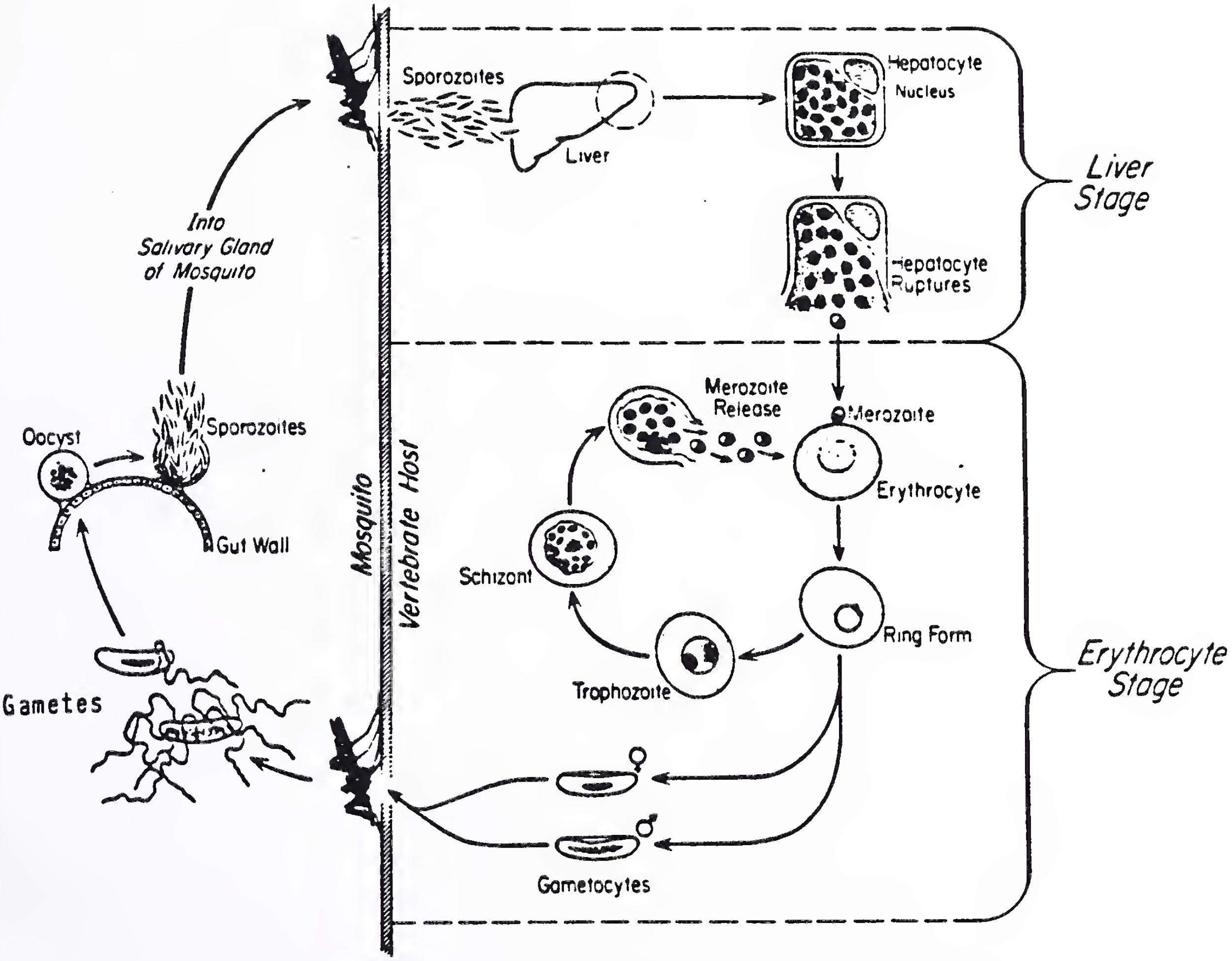


Figure 1. The life cycle of Plasmodium falciparum.

into the circulation. Within one half hour these released merozoites invade erythrocytes and initiate a new intra-erythrocytic cycle.

By an unknown mechanism, a subpopulation of invading merozoites enters a sexual cycle resulting in the formation of male and female gametocytes. If a female Anopheles ingests gametocytes during a blood meal, male and female gametes form and then join to make a zygote in the mosquito's stomach cavity. The zygote develops there into a motile ookinete form which penetrates the stomach wall and grows into an oocyst. Several hundred to one thousand sporozoites develop in each oocyst. These are liberated when the mature oocyst bursts open one to three weeks after gametocyte ingestion. The released sporozoites are dispersed throughout the mosquito's body. Those which come into contact with salivary gland tissue penetrate it and lodge in the acinal ducts. If the mosquito bites another human, it can inject sporozoites along with saliva into that person, thereby transmitting the parasite and possibly initiating a new infection.

The asexual erythrocytic cycle of parasite development is associated with the chills, fever, sweats, and related fever symptoms which characterize an acute attack of malaria. These paroxysms occur when schizonts rupture and release both merozoites and antigenic proteins that are by-products of intra-erythrocytic parasite development (4).

The higher morbidity and mortality rates associated with acute attacks and complications (e.g. massive hemolysis, renal failure, and cerebral malaria) of _P. falciparum_ versus the three other human malarias are due to at least two distinct properties of this species. First, _P. falciparum_ merozoites can invade a broader spectrum of erythrocytes than the other species (i.e. not only reticulocytes) (5). This ability allows _P. falciparum_ to produce higher parasitemias (concentration of asexual parasites per unit volume of blood) and thus greater degrees of erythrocyte alteration and destruction. Second, _P. falciparum_ causes infected erythrocytes to be sequestered in the microcirculation (6). Sequestration reduces contact with the spleen , a major site of parasite destruction, and places merozoites in regions of the vasculature where blood flow is reduced. This provides slower moving erythrocytes for merozoites to contact and attach to, and also leads to vaso-occlusion.

Several targets for potentially useful interventions emerge from a basic understanding of the _P. falciparum_ life cycle. Natural disease transmission would be abolished if gametocyte formation were blocked or if mosquitoes were prevented from harboring parasites or biting humans. In these situations either gametocytes would not be introduced into mosquitoes or sporozoites would not be introduced into man. Human infection would be prevented if sporozoites were

destroyed or their development within hepatocytes inhibited once introduced into the body. Human disease would either be prevented or eliminated if erythrocyte invasion were blocked. Merozoites and specific merozoite-erythrocyte interactions would thus be targets for interventions designed to reduce malarial disease.

A MICROSCOPIC VIEW OF INVASION

The physical events of erythrocyte invasion have been documented in light microscopic studies with *P. falciparum* (7) and both light and electron microscopic studies with the simian parasite *P. knowlesi* (8-13). The observations of Dvorak *et*. *al*. (8) and Aikawa *et*. *al*. (9) led to the realization that invasion is a brief but complex process. For descriptive purposes the process has been divided into the following phases: 1) recognition and attachment, 2) junction formation, 3) erythrocyte membrane invagination, and 4) erythrocyte membrane closure and parasitophorous vacuole formation. These phases are represented schematically in Figure 2. Less than 30 seconds is required for completion of all four phases (8).

Invasion begins as a host erythrocyte's life ends with rupture of a mature schizont and release of its merozoites. Individual *P. knowlesi* merozoites are ellipsoidal in shape and measure about 1.5 µm by 2.0µm (10). The anterior or apical end is distinguished by a small protuberance and a

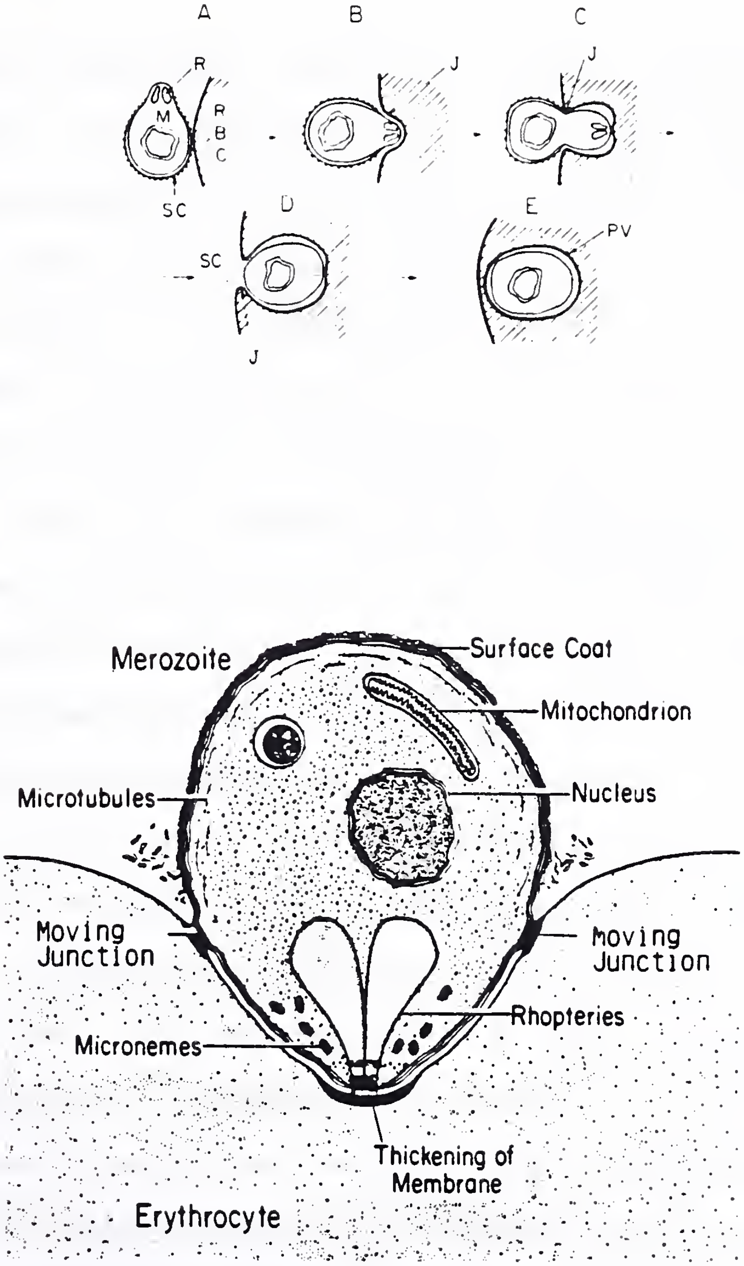


Figure 2. The four major phases of merozoite invasion into an erythrocyte (top) and a close-up view of a merozoite during phase three of invasion (bottom). Compiled from the results of Dvorak *et. al.* (8) and Aikawa *et. al.* (9). (A) Recognition and initial attachment of the merozoite to the erythrocyte. If the attachment does not occur via the apical end of the merozoite (B), reorientation occurs. (C) Junction formation and the beginning of invagination. (D) Erythrocyte membrane invagination. (E) Closure of the erythrocyte membrane and completion of parasitophorous vacuole formation. M, merozoite; RBC, erythrocyte; J, merozoite-erythrocyte junction; SC, surface coat; PV, parasitophorous vacuole.

series of organelles including paired rhoptry bodies and micronemes and microspheres. A series of ducts extend from the rhoptries to the apical surface. The nucleus is situated near the posterior end of the merozoite. A uniform surface coat of ~20nm thickness covers the entire merozoite (12) and is comprised of T- or Y-shaped bristle-like structures oriented perpendicularly to the outer membrane (10). Free P. knowlesi and P. falciparum merozoites are not cabable of locomotion, but they do exhibit bending and generalized contractile movements (7,8). Because of this lack of directed movment, the meeting of a merozoite and an erythrocyte appears to occur by chance.

Extensions of the bristle-like structures on the merozoite surface permit long-distance (60nm or more) contact between a merozoite and erythrocyte (10). This long-distance attachment may be the earliest event in the recognition and attachment phase. If the merozoite's apical end is oriented towards the erythrocyte, a closer attachment characterized by thicker bristles of 20nm length occurs immediately (Figure 2B) (10). If initial contact is not made at the apical end, the merozoite reorients itself while maintaining its long-distance attachment to the erythrocyte (Figure 2A) (10). In either instance the erythrocyte bends spasmodically for 5-10 seconds following merozoite attachment (7,8).

The entry phase begins as the membranes of the two

cells at the apical end attachment site form a region of close apposition (~10nm separation) called the junction (9). The erythrocyte membrane quickly becomes depressed and thickened in this region (Figure 2, bottom). One or more vacuoles or channels may appear in the erythrocyte cytoplasm under the junction (9). Entry proceeds by a progressive invagination of the erythrocyte membrane (Figure 2C). The pocket which forms during invagination will become a membrane surrounding the merozoite called the parasitophorous vacuole.

Invagination is accompanied by movement of the junction from the point of apical end attachment to the edges of the erythrocyte opening (9,11) (Figure 2C). Since electron micrographs always show junction regions at both edges of the erythrocyte opening, it appears that the junction completely encircles the merozoite while moving over it. The membrane surfaces between the moving junction and initial attachment site are not joined, indicating that the junction migrates over the merozoite circumferentially as would a ring (9). The merozoite surface coat is not observed inside the forming parasitophorous vacuole. When the merozoite is completely interiorzed, the junction comes together over the posterior end of the merozoite and closes like an iris diaphragm (9) (Figure 2D and E). This restores the defect in the erythrocyte generated by membrane invagination and completes parasitophorous vacuole

formation.

In electron micrographs the forming parasitophorous vacuole appears to be a continuation of the erythrocyte membrane (9). However, freeze fracture studies (11,13) have shown that the inner layer of the vacuolar membrane has a markedly reduced density of intramembranous particles relative to the erythrocyte membrane, and biochemical studies (14) have demonstrated that two erythrocyte membrane enzymes, NADH oxidase and ATPase, are in the opposite orientation in the vacuole than would be expected from simple erythrocyte membrane invagination. While it appears that the parasitophorous vacuole originates from the erythrocyte membrane, it is possible that some of it may derive from the merozoite (11).

Although the mechanism of merozoite entry is unknown, one of the more widely held hypotheses is that the apical end organelles secrete a substance into the erythrocyte during invasion which disrupts the membrane (12,15). This hypothesis is based upon the observation that the micronemes disappear and the rhoptries lose their electron-dense appearance during erythrocyte membrane invagination (12). The electron-dense rhoptry material appears to be largely proteinaceous (15), and may consist of a histidine-rich protein first identified in cytoplasmic granules of the duck parasite _P. lophurae_ (16). Indirect autoradiographic evidence suggests this protein may be located in or near the

rhoptries of _P. knowlesi_ (17). A similar histidine-rich protein in _P. falciparum_ has also been described (18). While the histidine-rich protein may be a critical factor for entry, its function and importance in this process have not yet been established.

The observation of longer (60nm) and shorter (20nm), thicker merozoite surface filaments in electron microscopic studies supports a mechanism involving two different attachments at the beginning of invasion (10). The 60nm filaments may facilitate long-distance attachment and the 20nm filaments a separate, short-distance attachment. Since the merozoite surface appears to be covered by anionic glycoproteins (10), these attachments could take place between surface proteins and/or saccharides on the interacting cells. Because the merozoite probably needs to be affixed firmly in order to continue with junction formation and entry, it seems likely that some attachments are made to erythrocyte surface molecules associated with the cytoskeleton. Among the erythrocyte surface molecules meeting this criterion is glycophorin A, which has an indirect connection through its association with band 3 protein (19) and possibly a direct connection to the cytoskeleton through band 4.1 (20,21).

ERYTHROCYTE DETERMINANTS IN *P. FALCIPARUM* INVASION

Species Specificity and the Erythrocyte Receptor Hypothesis

The earliest clues to the nature of the biochemical events of erythrocyte invasion came from experiments designed to determine the basis of *Plasmodium* host specificity. McGhee (22,23) was the first to hypothesize that differences of the outer surface of the erythrocyte membrane were responsible for restricting *Plasmodium* species to a limited number of hosts. McGhee based his hypothesis on the observation that *P. lophurae*, an avian malaria which infects ducks and chickens, infected duck erythrocytes introduced into chicken embryos at a significantly greater rate than the more numerous chicken erythrocytes (22). Using *P. knowlesi*, Butcher *et*. *al*. (24) showed that susceptibility to invasion *in vitro* correlated only with properties of erythrocytes, and that merozoites bound only to erythrocytes from susceptible hosts (e.g. merozoites bound to monkey and human erythrocytes but not hamster, rabbit, or guinea pig erythrocytes). These results indicated that a receptor for merozoites was located on the erythrocyte membrane, and that specific interactions between the surfaces of the erythrocyte and merozoite were necessary for invasion to occur.

After obtaining preliminary evidence indicating that the *P. knowlesi* receptor on human erythrocytes was not one of the sialoglycoproteins (SGP) (25), Miller *et*. *al*. (26)

performed a comparative study with P. knowlesi and P. falciparum to determine the basic differences in the receptors for these two parasites. Their experiments were made possible by a new in vitro culture technique for P. falciparum developed by Trager and Jensen (27). Because parasites could be cultured continuously, Miller's group was able to add parasite-infected erythrocytes to cultures of test erythrocytes and determine how efficiently the released merozoites infected test cells.

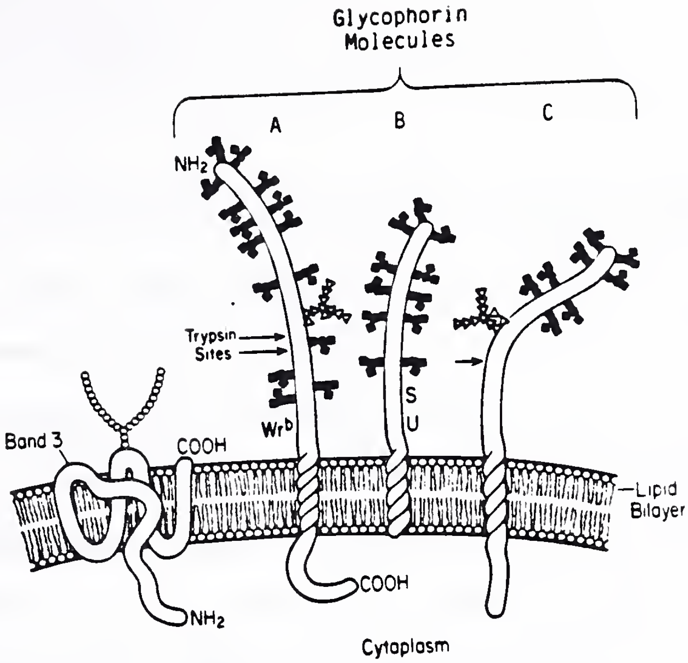
Miller's group first compared susceptibility to invasion in human erythrocytes lacking different blood group antigens. They found that while P. falciparum invaded erythrocytes lacking the Duffy antigens [Fy(a-b-)] as efficiently as normal erythrocytes, it invaded erythrocytes totally lacking glycophorin A [En(a-) (28,29,30)] to a level only 39-47% of normal erythrocytes. [It should be noted that En(a-) erythrocytes also have increased glycosylation of band 3 protein (29) and lack the Wright b (Wr^b) antigenic determinant.] Secondly, they observed that trypsin (1 mg/ml) or neuraminidase (25 U/ml) treatment of normal erythrocytes significantly reduced their susceptibility to P. falciparum but not to P. knowlesi invasion. The opposite result was obtained with chymotrypsin-treated (0.1 mg/ml) normal erythrocytes. Miller's group interpreted these results to mean that different proteins or glycoproteins were involved in the recognition of erythrocytes by the two

parasites. Knowing that trypsin removed portions of the SGP and neuraminidase removed sialic acid from the erythrocyte surface, they postulated that the SGP served as the *P. falciparum* receptor. Since En(a-) erythrocytes were only partially resistant to invasion, they thought glycophorin A could not be the sole receptor molecule.

The Human Erythrocyte Sialoglycoproteins

Before presenting the results of experiments which were performed to assess more precisely the role of the SGP, the essential features of these molecules will be reviewed briefly. When normal erythrocyte membranes are dissolved in an excess of sodium dodecyl sulfate and then electrophoresed on a polyacrylamide gel (SDS-PAGE), at least seven bands representing sialic acid-rich glycoproteins can be visualized by periodic acid Schiff (PAS) staining (reviewed in 31). These bands are comprised of at least four distinct sialoglycoproteins. The bands have been given a variety of names by different investigators, but will be referred to here according to Furthmayr (32) as glycophorin A (α_2 and α, PAS-1 and PAS-2), glycophorin B (δ_2 and δ, PAS-3) and glycophorin C (β and γ, PAS-2′). These are shown in schematic form in Figure 3.

The major sialoglycoprotein, glycophorin A (GpA), is an integral membrane glycoprotein of 131 amino acids and 31,000



GENERALIZED STRUCTURES OF THE CARBOHYDRATE UNITS OF NORMAL AND VARIANT RED CELL GLYCOPHORINS

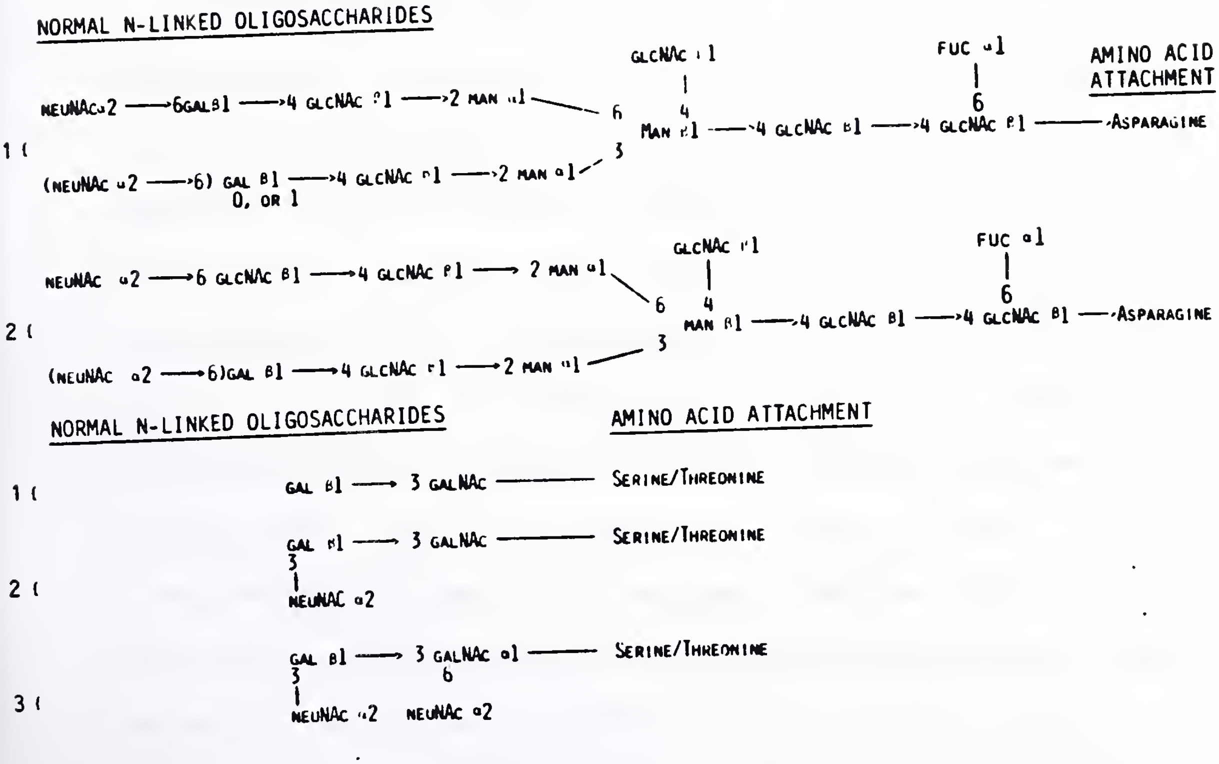

NORMAL N-LINKED OLIGOSACCHARIDES
AMINO ACID ATTACHMENT
1 (
NEUNAc α2 → 6 GAL β1 → 4 GLCNAc β1 → 2 MAN α1 — 6
(NEUNAc α2 → 6) GAL β1 → 4 GLCNAc β1 → 2 MAN α1 — 3
0, OR 1
GLCNAc β1
4
MAN β1 → 4 GLCNAc β1 → 4 GLCNAc β1 → ASPARAGINE
FUC α1
6
2 (
NEUNAc α2 → 6 GLCNAc β1 → 4 GLCNAc β1 → 2 MAN α1 — 6
(NEUNAc α2 → 6) GAL β1 → 4 GLCNAc β1 → 2 MAN α1 — 3
GLCNAc β1
4
MAN β1 → 4 GLCNAc β1 → 4 GLCNAc β1 → ASPARAGINE
FUC α1
6
NORMAL N-LINKED OLIGOSACCHARIDES
AMINO ACID ATTACHMENT
1 (
GAL β1 → 3 GALNAc — SERINE/THREONINE
2 (
GAL β1 → 3 GALNAc — SERINE/THREONINE
3
NEUNAC α2
3 (
GAL β1 → 3 GALNAc α1 — SERINE/THREONINE
3
6
NEUNAc α2
NEUNAc α2

Figure 3. Glycophorins A, B, and C in the human erythrocyte membrane (top) and the structure of the two types of normal N-linked oligosaccharides and three types of normal O-linked oligosaccharides attached to the glycophorins (bottom). Wr^b, Wright b antigen; S, U, S and U antigens; ➧, trypsin cleavage site; ■, O-linked oligosaccharides; △, N-linked oligosaccharide; -OO-, band 3 oligosaccharide, NEUNAc, N-acetyl-neuraminic acid; GLCNAC, N-acetyl-glucosamine; GALNAC, N-acetyl-galactosamine; FUC, fucose; MAN, mannose; GAL, galactose.

molecular weight (33) which probably exists in the membrane as a dimer (34) because of hydrophobic interactions at its intramembranous segment. Estimates of the number of copies/cell vary between 5 x 10^5 (35) and 2 x 10^6 (36). GpA has a tripartite structure consisting of a glycosylated, NH_2-terminal segment of 64 amino acids, a hydrophobic segment of 32 nonpolar amino acids, and a relatively hydrophillic, COOH-terminal segment of 35 amino acids (see Figure 4).

Approximately 60% of the dry weight of GpA is attributable to carbohydrate, all of which is located on the 15 O-glycosidically linked tetrasaccharides and 1 N-glycosidically linked oligosaccharide attached to the NH_2-terminal segment (33). The structures of the two types of normal N-linked oligosaccharides (37) and three types of normal O-linked tetrasaccharides (38) are shown in the bottom section of Figure 3. These oligosaccharides are not unique and can be found in other types of soluble glycoproteins. Each GpA monomer carries 32 sialic acid (N-acetyl-neuraminic acid) moieties, giving both itself and the entire erythrocyte surface a net negative charge.

When erythrocytes are treated with trypsin, GpA molecules are cleaved in their NH_2-terminal segments. Two peptides named T1 and T2, both of which retain their attached oligosaccharides, are released (32). Trypsin treatment of purified GpA yields these same two

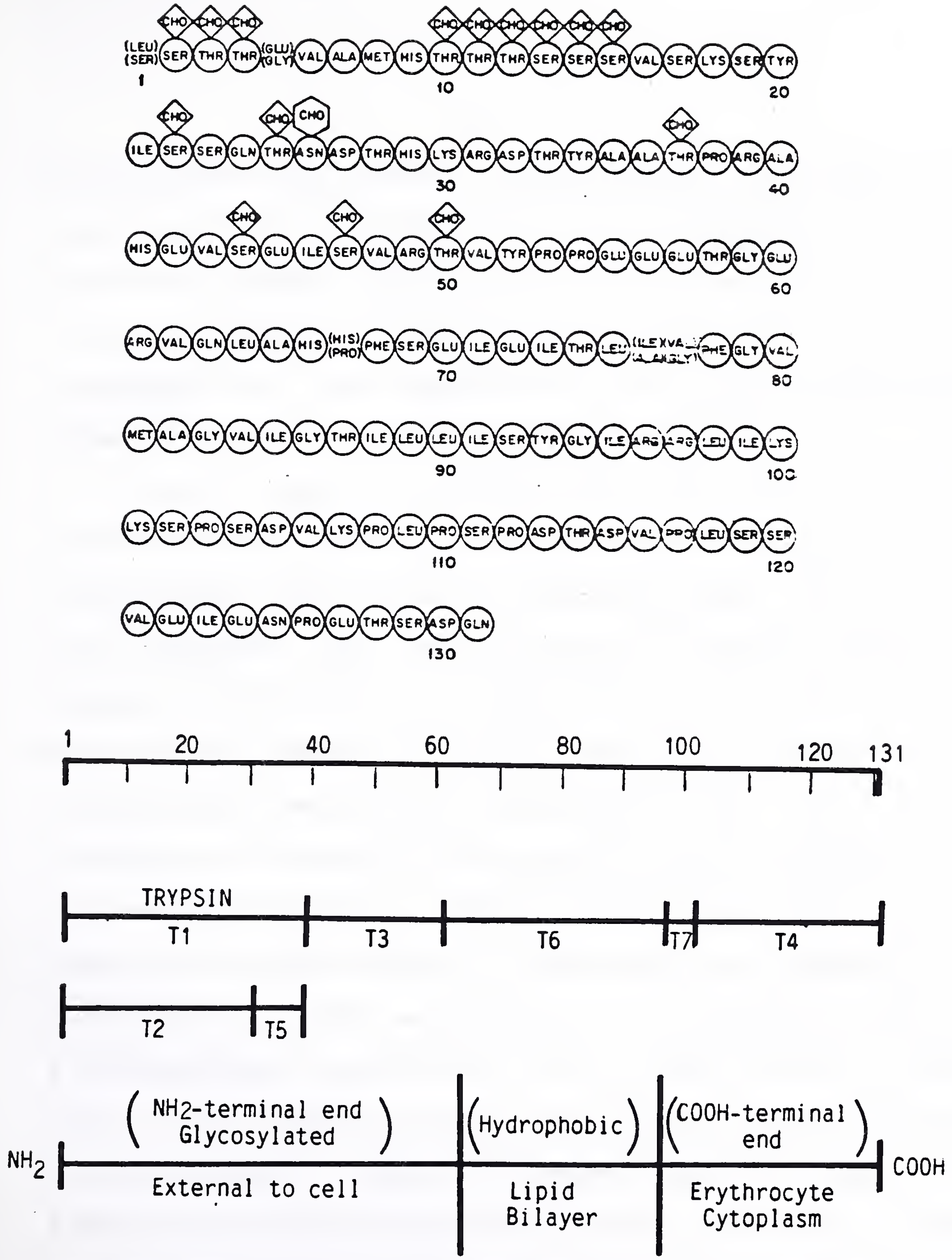

CHO
(LEU) (SER) SER THR THR (GLU) (GLY) VAL ALA MET HIS THR THR THR SER SER SER VAL SER LYS SER TYR
1
10
20
ILE SER SER GLN THR ASN ASP THR HIS LYS ARG ASP THR TYR ALA ALA THR PRO ARG ALA
30
40
HIS GLU VAL SER GLU ILE SER VAL ARG THR VAL TYR PRO PRO GLU GLU GLU THR GLY GLU
50
60
ARG VAL GLN LEU ALA HIS (HIS) (PRO) PHE SER GLU ILE GLU ILE THR LEU (ILE)(VAL) (ALA)(GLY) PHE GLY VAL
70
80
MET ALA GLY VAL ILE GLY THR ILE LEU LEU ILE SER TYR GLY ILE ARG ARG LEU ILE LYS
90
100
LYS SER PRO SER ASP VAL LYS PRO LEU PRO SER PRO ASP THR ASP VAL PRO LEU SER SER
110
120
VAL GLU ILE GLU ASN PRO GLU THR SER ASP GLN
130
1
20
40
60
80
100
120
131
TRYPSIN
T1
T3
T6
T7
T4
T2
T5
NH2-terminal end
Glycosylated
Hydrophobic
COOH-terminal end
NH2
COOH
External to cell
Lipid Bilayer
Erythrocyte Cytoplasm

Figure 4. Amino acid sequence of human erythrocyte glycophorin A (top) and a schematic representation of the tryptic peptides of glycophorin A and their location relative to the erythrocyte membrane (bottom). Reprinted and redrawn from Tomita and Marchesi (33). CHO, oligosaccharide; ◇, O-glycosidic linkage; ⬡, N-glycosidic linkage.

glycopeptides plus several others (39,40). The amino acids (aa) comprising the six largest tryptic peptides of purified GpA are as follows: aa 1-39=T1, aa 1-31=T2, aa 40-61=T3, aa 101 or 102-131=T4, aa 32-39=T5, and aa 62-97=T6 (40). The arrangement of these sequences on intact GpA is represented schematically in the bottom half of Figure 4.

Several types of erythrocytes with alterations or deficiencies of GpA have been described. Homozygous En(a-) erythrocytes, which possess no detectable GpA, have already been discussed. AG erythrocytes have a hybrid glycophorin molecule consisting of approximately 56 aa of the NH_2-terminal portion of GpA connected to the portion of glycophorin B inserted into the membrane (41). NH_2-terminal portions of this hybrid glycophorin can be cleaved off by treatment with trypsin. MgMg erythrocytes have a single amino acid substitution of threonine ⟶ asparagine at position 4 of the GpA sequence (42). This mutation prevents glycosylation at amino acids 2, 3, and 4 of GpA, so these cells are lacking three of the fifteen O-linked oligosaccharides. McM erythrocytes have an amino acid substitution either at position 1 or 5 on GpA (42). Since the expression of M or N blood group activity depends on the nature of the amino acids at these two positions (39,43), McM erythrocytes are intermediate between normal M or N antigenic groups.

Glycophorin B (GpB) is one of the minor erythrocyte

sialoglycoproteins and is not as well defined as GpA. It appears to be smaller than GpA, probably because it lacks a cytoplasmic segment (32). Erythrocytes are believed to possess approximately 70,000 copies of GpB/cell (32). Furthmayr (39) has shown that the first 23 to 25 NH_2-terminal amino acids of GpA (blood group N) and GpB are identical, including the sites of oligosaccharide attachment. GpB differs from GpA, however, in at least three ways: 1) it lacks GpA's N-linked oligosaccharide attached to asparagine at position 26, 2) it has a sequence on the COOH-terminal side of this position which is markedly different than GpA (39), and 3) it is not cleaved during trypsin treatment of erythrocytes.

GpB is known to carry at least three antigens, the S and s antigens and the U antigen (31). The U antigen has not been characterized and its location is unknown. S and s antigenic activity are related to the amino acids and attached oligosaccharides at positions 25-31 of GpB (44). Erythrocytes lacking these three antigens, called S-s-U(-), have been described (45). They either lack GpB completely or possess GpB molecules which are deficient in carbohydrate moieties essential for Ss antigenic activity (46).

Glycophorin C (GpC) is the other minor sialoglycoprotein on erythrocytes. Unlike GpA and GpB, whose PAS-staining bands on SDS-PAGE represent monomers or dimers of a single type of sialoglycoprotein, GpC is

comprised of two different sialoglycoproteins, β and γ (31). Approximately 35,000 copies of GpC exist per erythrocyte (32), making it the least prevalent of the glycophorins. Although GpC appears to have an N-linked oligosaccharide (32) and O-linked oligosaccharides similar to those found on GpA (31), its NH_2-terminal amino acid sequence (specifically the β sialoglycoprotein) is completely different (47). GpC on normal erythrocytes is susceptible to cleavage by trypsin. The glycopeptide liberated is called DT1 and represents aa 1-47 (47). Two individuals lacking the Gerbich (Ge) antigen plus β , γ , and a third minor sialoglycoprotein designated β_1 have been described (48). Because some of the erythrocytes circulating in these Ge(-) individuals are elliptocytic, Anstee *et. al.* (48) have proposed that one or more of these sialoglycoproteins have a role in maintaining the shape of the erythrocyte.

Two other variant erythrocytes which have alterations in both GpA and GpB have been described. *Tn* erythrocytes possess decreased amounts of sialic acid (approximately 46% of normal) and galactose (49) partly because of an acquired defect in β-3-D-galactosyltransferase (50). This defect exposes a cryptantigen on erythrocytes called the Tn antigen. Affected individuals have Tn antigen on as few as 5% or as many as 90% of their erythrocytes (51). *Cad* erythrocytes have a normal sialic acid content but possess a pentasaccharide containing a N-acetyl-galatosamine added in

a β(1-4) linkage to the galactose present in the normal O-linked tetrasaccharides of GpA and GpB (52).

While the function of the SGP is still unknown, they are known to carry a variety of blood group antigens (M and N on GpA, N, S, s, U on GpB) and to serve as a binding site for myxoviruses (GpA) (53). One of the chief difficulties in assigning specific functions to these molecules is the fact that individuals whose erythrocytes lack GpA [En(a-)], GpB [S-s-U(-)], or GpA and GpB [homozygous M^k erythrocytes, (54)] are all hematologically normal (31).

The Role of Glycophorins in *P. falciparum* Invasion

The two experimental approaches most commonly used to study the role of glycophorins (Gps) in *P. falciparum* invasion have been invasion-inhibition experiments and merozoite-binding studies. In invasion-inhibition experiments, exogenous substances or altered erythrocytes are introduced into parasite cultures and their effect on invasion determined by comparing the percentage of erythocytes invaded in test versus control cultures. All of the following materials have been used in invasion-inhibition and/or merozoite-binding studies:

1) crude glycophorin preparations and purified Gps in solution
2) liposomes carrying Gps and purified GpA
3) glycopeptide and peptide fragments of GpA
4) erythrocytes altered by enzymatic treatments or genetically-defined membrane defects
5) naturally occurring and synthetic saccharides
6) polyclonal and monoclonal antibodies against GpA

Weiss _et_. _al_. (55) were the first to evaluate the effect of soluble human SGP on _P._ _falciparum_ invasion _in vitro_. They added purified GpA to cultures of synchonized, schizont-infected erythrocytes and assessed the ability of merozoites to invade uninfected erythrocytes. GpA had no inhibitory effect at <200 μg/ml concentration, but did cause 28% inhibition at 500 μg/ml. The inhibition observed, presumably due to a competitive effect, was felt by these workers to be too minor to provide support for GpA as a receptor.

Since the report of Weiss _et_. _al_. (55), five more sets of experiments with both mixed and purified glycophorins have been published (56-60). Concentrations varying from 0.05-1.0 mg/ml were required to achieve 50% inhibition levels. Of the preparations tested, Gps and GpA alone were the most inhibitory. However, in the hands of Vanderberg _et_. _al_. (60), there was no difference between Gps and individual fractions of GpA, GpB, and GpC. Schulman _et_. _al_. (59) documented the inhibitory specificity of the Gps fraction from their erythrocyte membrane preparations by showing that other fractions (e.g. the band 3 membrane fraction) produced no inhibition.

Instead of using soluble Gps during the entire incubation period needed for schizont rupture and merozoite invasion, Perkins (61,62) incubated soluble Gps with isolated merozoites before adding them to uninfected

erythrocytes. Purified GpA caused 79% inhibition at only 10 µg/ml, while 100% inhibition was achieved with 50 µg/ml. GpB inhibited invasion ~90% at 200 g/ml. Perkins also showed free merozoites possess a saturable binding site for ^{125}I-glycophorins (mixture), providing the first direct evidence that merozoites could bind to their proposed erythrocyte receptors (62).

Although the methods employed both to prepare glycophorin and to assess invasion inhibition varied in the aforementioned studies, all the authors concluded that Gps were likely to be at least a component of the erythrocyte receptor (56-62). Based on her results, Perkins (62) suggested that GpB could function as a receptor molecule (although not as effectively) in addition to GpA in normal erythrocytes. Vanderberg et. al. (60), who found that GpA, GpB, and GpC were equally good inhibitors, suggested that they could all serve as receptor molecules.

A second technique used to study the inhibitory activity of human Gps was the introduction of Gps-containing liposomes into invasion assay systems (57,58,61). Perkins (61) prepared liposomes by separately adding the aqueous phase, interphase, and lipid/glycolipid phase of a chloroform:methanol extraction of erythrocyte membranes to liposomes. Of the three liposome preparations, only the one containing the aqueous phase material, where the majority of the GpA partitioned, caused significant inhibition. Breuer

et. al. (58) utilized egg lecithin liposomes to which either no proteins, Gps extracted as by Perkins (62), or a human erythrocyte band 3 fraction was added. They found that 6 to 7 times less Gps was required to give 50% inhibition when they were added in liposomes instead of in soluble form. Liposomes with no protein or the band 3 fraction had no significant inhibitory effect. In addition to supporting the results obtained with soluble Gps, Breuer's group felt their liposome data suggested that the environment surrounding the glycophorins could strongly influence invasion inhibition as well.

Based on the assumption that the glycophorins were the merozoite receptors, several investigators attempted to determine which domains of these molecules were critical for invasion. These domain analyses were performed by determining the inhibitory effects of enzymatically-derived glycophorin peptides on invasion. All the tryptic peptides of GpA shown in Figure 4 plus DT1 (aa 1-47) from GpC and several other incompletely characterized peptides have been used in these studies.

Three groups investigated the role of carbohydrate-rich domains by using the tryptic glycopeptides T1, T2, T1+T2, T5, and DT1 in invasion-inhibition experiments (58,60,62). The carbohydrate-rich domains were believed to be critical for invasion based on the earlier experiments of Miller et. al. (26) and studies to be described subsequently. The

results of these domain studies are shown in Table 1. None of these glycopeptides caused a significant degree of inhibition except for the T1 of Perkins (62), which caused 76±11% inhibition at 100 µg/ml.

It is difficult to compare the results of these groups because different methods for purifying the glycophorins and assessing invasion inhibition were used. Perkins' result with T1 could be due to the fact that her original Gps preparation was significantly more inhibitory than the one used by Vanderberg _et_. _al_. (60) or Breuer _et_. _al_. (58). Vanderberg, however, suggested that the unexpectedly low level of inhibition observed with these fragments by his and Breuer's group was related to the dissociation of NH_2-terminal segments from hydrophobic segments of GpA. He based this idea on work done by Furthmayr (39), who showed that these glycopeptides had a three- to four-fold decrease in MN blood group serological activity per mole compared with intact GpA. Because the hydrophobic segment is missing, these fragments cannot form micelle-like structures in aqueous solution (63), a phenomenon which produces clustering of the NH_2-terminal oligosaccharides and a superior target for anti-M and N antibodies. Thus, sialoglycopeptides may not present the preferred cluster of oligosaccharide units to the merozoite in order to serve effectively as competitive inhibitors.

Perkins (62) and Breuer _et_. _al_. (58) also used

Table 1. Inhibitory Effect of Human Erythrocyte Glycophorins and Glycophorin Peptides on *P. falciparum* Invasion

Study	Glycophorin Preparation Added	Protein Concentration, ug/ml	Percent Inhibition vs. Control
Breuer *et*. *al*. (59)	None (Control)	0	0
	Gps	100	50±5
		200	84±6
	T1+T2	1200	17±7
	T5	700	0
	T6	200	70±4
Vanderberg *et*. *al.* (61)	None (Control)	0	0
	Gps	100	25±5
		200	50±7
	GpA	100	30±4
	GpC	100	27±5
	T1	1000	24±3
	T2	1000	15±4
	DT1	1000	8±3
Perkins (63)	None (Control)	0	0
	Gps	50	85±7
		100	100
	GpA	50	77±11
		100	100
	T1	100	76±11
	T2	100	13±5
	T3	200	25±6
	T4	200	0±2
	T5	200	52±13
	T6	100	67±4

[a] Percent inhibition represents 100% (control invasion rate) minus the invasion rate (percent of cells infected with ring form parasites) in test cultures ± standard deviation.

glycophorin peptides to assess the importance of carbohydrate-poor domains for invasion. Tryptic peptides T3, T4, and T6 were used in these experiments (see Figure 4). As shown in Table 1, only T6 appeared to possess significant inhibitory activity. Both Perkins and Breuer et. al. suggested that the hydrophobic segment of GpA could be a merozoite binding site. This interpretation is complicated by the fact that non-inhibitory, soluble proteins can be transformed into potent invasion inhibitors by derivitization with hydrophobic side groups (64). Thus, the hydrophobic nature of T6 and not a binding site for merozoites may have caused the inhibition observed.

Experiments with human erythrocytes altered either by enzymatic treatment or genetically-defined membrane defects have provided strong evidence in support of a receptor role for the Gps. Trypsin (26,65,66,67), neuraminidase (26,61,67), and pronase (67) treatment of erythrocytes have all been shown to decrease invasion significantly. Chymotrypsin, reported to be without effect at 0.1 mg/ml by Miller et. al. (26), decreased susceptibility to invasion by >75% when used at concentrations of 1 and 10mg/ml (66). While these enzymes are known to modify glycophorins on the erythrocyte surface, their effects are insufficiently selective to provide specific information on the importance of particular glycophorin molecules or domains for invasion. When used to treat variant erythrocytes, however, these

enzymes have provided important clues about individual glycophorin species.

Glycophorins A, B, and C have all been implicated as receptors from studies done with erythrocytes possessing genetically-defined and acquired membrane defects (26,66,68-72). Table 2 summarizes the results of invasion-inhibition studies performed with the eight types of variant erythrocytes described in the previous section. A ninth type of variant erythrocyte called Wright b negative [Wr^b(-)] was also once thought to be highly resistant to invasion (69). These erythrocytes lack the Wr^b antigen because of an undetermined alteration between amino acids 62 and 72 of GpA (73). Follow-up studies (74-77) have demonstrated that Wr^b(-) erythrocytes are invaded normally by _P. falciparum in vitro_, so this variant is not included in Table 2.

Although Miller _et_. _al_. (26) were the first to publish results showing En(a-) erythrocytes are partially resistant to invasion, Pasvol _et_. _al_. (68) were the first to argue strongly that this reduced susceptibility supported Perkins' (61) proposal that GpA was a merozoite receptor. Pasvol tested En(a-) erythrocytes from three different donors and found the mean percentages of invasion for these erythrocytes were 8 ± 7%, 12 ± 1%, and 48 ± 5% of normal En(a+) erythrocytes. He also demonstrated that _P. falciparum_ could grow equally well in En(a-) and En(a+)

Table 2. Results of Invasion Inhibition Experiments Performed with Genetically-defined and Acquired Variant Erythrocytes

Variant Erythrocyte	Description	Susceptibility in Range of % Control Invasion	Study
En(a-)	Complete lack of GpA	39-46	26
		7-57[a]	69
		38	72
AG	Hybrid glycophorin with ∼56 aa of GpA at NH_2-terminal end and remainder GpB	25-70	69
AG, trypsin treated	Removes 39 aa at NH_2-terminal end of GpA	15-31(36-70)[b]	69
MgMg	Lacking 3 O-linked oligosaccharides at positions 2-4 on GpA	Normal	72
McM	Single aa substitution making GpA intermediate between normal M and N antigenic groups	Normal	72
S-s-U(-)	Complete lack of GpB or possess defective oligosaccharides on GpB	87	26
		69-75	70
		63-100	71
		30-57	67
S-s-U(-), trypsin treated	Removes 39 aa and 47 aa from NH_2-terminal ends of GpA and GpC, respectively	5-7(37-39)[b]	70
		2-8(11-31)[b]	71
		7-9(5)	67
Ge(-)	Complete lack of SGP β, β_1, and γ	51-63	73
Ge(-), trypsin-treated	Removes 39 aa at NH_2-terminal end of GpA	17-29(27-38)[b]	47

Table 2. (Continued)

Variant Erythrocyte	Description	Susceptibility Range of % Control Invasion	Study
Tn	Inadequate glycosylation of GpA and GpB. 46% of normal sialic acid content	100	26
		6-10	70
		2-8	72
Cad	Additional N-acetyl-galactosamine on O-linked oligosaccharides of GpA and GpB	1-13	72

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[a] Three different erythrocyte sources were used. Two lacked only GpA and one lacked part of GpB as well.

[b] Numbers in parentheses are the range of percent invasion rates in trypsin-treated normal erythrocytes. These are included to control for the effects of trypsin treatment.

cells, and that anti-En^a antibodies could greatly reduce invasion into En(a+) cells in a specific fashion. Facer (66) showed that En(a-) cells were partially resistant to invasion and also argued that this supported an important role for GpA in invasion.

The results of invasion-inhibition experiments with S-s-U(-) and trypsin-treated normal S+s+U(+) erythrocytes suggest GpB may also function as a _P. falciparum_ receptor molecule (66,69,70). S-s-U(-) erythrocytes have a partial resistance to invasion (Table 2). Pasvol _et_. _al_. (69) and Howard _et_. _al_. (70) have suggested a role for GpB based on the observation that trypsin-treated S+s+U(+) erythrocytes are intermediate in susceptibility between the more susceptible S+s+U(+) cells and the less susceptible S-s-U(-) cells. While S+s+U(+) erythrocytes possess intact Gps, trypsin-treated S+s+U(+) cells have only intact GpB (i.e. trypsin cleaves both GpA and GpC) and trypsin-treated S-s-U(-) cells have no intact Gps. Structural data showing GpA and GpB share identical NH_2-terminal sequences for 23 to 25 aa (39) and invasion-inhibition experiment results showing En(a-) erythrocytes are still partially susceptible have been cited in support of GpB as well (69).

Both Tn and Cad erythrocytes have been demonstrated to be significantly resistant to invasion (69,71) (Table 2). The normal susceptibility of the Tn erythrocytes studied by Miller _et_. _al_. (26) (Table 2) could have been due to the

fact that their Tn donor had a relatively low percentage of erythrocytes affected by this acquired glycosylation defect (71). When approximately 90% of the erythrocytes used in test cultures were affected, invasion rates were <10% of normal (69,71). Because both GpA and GpB are altered in Tn, Cad, and AG erythrocytes, it is not possible to infer a specific role for either glycophorin from these results. MgMg and McM erythrocytes, which both have minor carbohydrate changes at the NH_2-terminal end of GpA, are normally invaded (Table 2).

Pasvol et. al. (72) recently demonstrated that erythrocytes lacking GpC [Ge(-)] are partially resistant to invasion (Table 2). Invasion of Ge(-) erythrocytes was approximately 57% of normal erythrocytes. Trypsin treatment of Ge(-) erythrocytes, like treatment of S-s-U(-) erythrocytes, reduced invasion further. Although the amino acid sequence of GpC is different from that of GpA and GpB, GpC's oligosaccharides appear to be similar (47). While these results have been used to support a role for GpC in merozoite invasion (72), it should be remembered that at least some Ge(-) erythrocytes are elliptocytic and may have cytoskeletal alterations as well.

Carbohydrates on the erythrocyte surface have been considered important to the erythrocyte-merozoite interaction ever since Miller et. al. (26) first showed that neuraminidase treatment greatly reduced invasion. The

binding of P. falciparum to erythrocyte carbohydrates would not be an unusual mechanism of cell-cell interaction, since other parasites (78,79) and some viruses (80) and bacteria (81-83) bind to receptors comprised of carbohydrate molecules. In these interactions, which resemble the binding of plant lectins such as wheat germ agglutinin and soy bean agglutinin, carbohydrates appear to serve as bridging ligands.

One of the most commonly employed methods for studying lectin-like binding phenomena is the use of exogenous saccharides in competitive inhibition experiments. The complex structure of the oligosaccharide side chains attached to GpA (Figure 3) has prompted the testing of more than 40 different saccharide species for their ability to inhibit invasion (55,56,58,60,62,70,84-86). Table 3 lists the 13 molecules which have been shown by investigators to cause >40% inhibition at <100mM in invasion assays with schizont-infected erythrocytes. Those molecules known to cause a toxic or reversibly retarding effect on developing parasites are excluded [e. g. glucosamine-HCL (55), fucose (55), and mannose (86)]. The inhibitory effect of N-acetyl-D-glucosamine (GlcNAc) was originally felt to be secondary to a toxic effect (55). Jungery et. al. (57), however, used both lower concentrations (<25mM) and GlcNAc linked to bovine serum albumin (BSA) to exclude toxicity and demonstrate a true inhibitory effect for this saccharide.

Table 3. Saccharides Causing Significant Inhibition (>40%) of *P. falciparum* Invasion When Added as Exogenous Competitors

Saccharide	Range of concentrations tested (mM)	Range of inhibition observed (% vs control)	Study
DGlcNAc	0.1-100	10-95	86
	2.5-30	6 to > 95	87
	2.5-40	8-90	61
	2.5-80	10-100	58
	5-100	2-100	56
	10-100	0-100	71
	25	69	59
NeuNAc	3.0	0	57
	10.0	17	59
	10-20	21-30	87
	2.5-25	0-52	58
	2.5-40	5-85	61
DGalNAc	5-80	7-70	58
	10-100	0-90	71
	100	0	56
Chitobiose	1-10	3-80	71
	2.5-30	52-85	87
	Not given	Negative	59
NeuNGc	2.5-40	10-95	61

Table 3. (Continued)

Saccharide	Range of concentrations tested (mM)	Range of inhibition observed (% vs control)	Study
NeuNAc-Lac	2.5-20	5-55	61
αDGlcNAcOMe	10-30	23-60	87
αDGalNAcOMe	10 and 30	26-61	87
Colomic Acid	0-2.0mg/ml	0-87	61
βDGlcNAc(1→4)αDManOMe	10-20	66-81	87

DGlcNAc, N-acetyl-D-glucosamine: NeuNAc, N-acetyl-neuraminic acid: DGalNAc, N-acetyl-D-galactosamine: Chitobiose, βDGlcNAc(1→4)DGlcNAc , NeuNGc, N-glycolylneuraminic acid: NeuNAc-Lac, N-acetyl neuramin-lactose: αDGlcNAcOMe, methyl 2-acetamido-2-deoxy-α, β-D-glucopyranoside: Colomic acid, NeuNAc polymer: βDGlcNAc(1→4)αDManOMe.

Subsequent studies with conjugated GlcNAc have supported these results (85,86).

Of the 13 saccharides listed in Table 3, DGlcNAc has often produced the greatest degree of inhibition in comparative studies by different investigators. An inspection of the oligosaccharide structures found on GpA (Figure 3) reveals that GlcNAc is a common terminal and core saccharide moiety. Exogenous DGlcNAc may inhibit merozoites from binding to glycophorins by competing with the GlcNAc found in glycophorin oligosaccharides. The other inhibitory saccharides presumably generate a similar type of competitive inhibition, but it should be noted that not all of the saccharides found in Table 3 are represented on glycophorin oligosaccharides. Also, some of the saccharides present on glycophorin, such as galactose, do not inhibit invasion when added to cultures (55,57,86). Until the three-dimensional structures of the glycophorin oligosaccharides are known, it will be difficult to explain why saccharides such as galactose do not inhibit invasion. Both terminal (e.g. NeuNAc and GlcNAc) and core [e.g. βGlcNAc(1$\rightarrow$4)DGlcNAc and βDGlcNAc(1$\rightarrow$4) αDMan] saccharides appear to play a role, however.

Conflicting results and differing invasion-inhibition assay methods have led to uncertainty in the literature concerning a role for sialic acid (N-acetyl-neuraminic acid, NeuNAc). Several groups showed that neuraminidase treatment

of erythrocytes significantly reduced invasion (26,61,70), but soluble, desialylated Gps did not appear to be less inhibitory than soluble, untreated Gps in most experiments (26,61,84). Earlier competitive inhibition experiments with NeuNAc suggested it was non-inhibitory (56,58,86) (Table 3), but Vanderberg _et_. _al_. (60) recently tested it over a wide range of concentrations and found the previous negative results to be attributable to insufficient amounts of NeuNAc added to cultures. Jungery _et_. _al_. (57) found similar results. Although one group has claimed sialic acid plays a very important role in invasion (60), all the groups investigating sialic acid state that it is not, by itself, sufficient for invasion. Several lines of evidence suggest that the negative charge imparted to the erythrocyte surface by sialic acid does not determine merozoite recognition (reviewed in 87).

Competitive inhibiton experiments similar to those performed with free saccharides have also been performed with saccharides coupled to BSA (57,85,86). Jungery _et_. _al_. (57) coupled GlcNAc and lactose to BSA by a p-aminophenyl spacer to prevent these saccharides from entering erythrocytes and producing toxic effects on developing parasites. While BSA alone and BSA-lactose caused no invasion inhibition from 10^{-6}M to 10^{-1}M, BSA-GlcNAc produced strong inhibition up to 10^{-5}M. Compared to GlcNAc alone, BSA-GlcNAc was approximately 100,000 times more effective

in producing inhibition. Schulman et. al. (85) prepared BSA conjugates of GlcNAc and several other saccharides with amide spacers and found BSA-GlcNAc was 84 times more effective than GlcNAc in producing identical levels of inhibition.

Hermentin et. al. (86) were unable to generate a marked enhancement of inhibitory activity using a BSA-GlcNAc congugate prepared with an aliphatic spacer. Suspecting that the nature of the spacer might influence a congugate's inhibitory ability, Hermentin's group coupled BSA and GlcNAc with a variety of different spacers and showed that the strength of inhibition correlated closely with the degree of hydrophobicity of the spacer. Using amide spacers they found the greatest enhancement in inhibition with a BSA-βDGal(1→3) αDGalNAc conjugate. This result suggests that the core structure of the O-linked oligosaccharides plays a role in merozoite recognition and attachment.

The ability of specific carbohydrates to inhibit invasion led to the idea that the interaction between merozoites and erythrocytes is lectin-like (57) and stimulated efforts to select for parasite proteins which bind to either GlcNAc (88) or Gps (88,89). Jungery et. al. (88) added parasite proteins to a column of GlcNAc coupled to Sepharose 4B beads and found that three proteins of molecular weight (MW) 140 kilodaltons (kd), 70kd, and 35kd bound specifically to the coupled sugar. The 140kd and 35kd

proteins plus two others of 210kd and 140kd also bound to Gps coupled to Sepharose beads. Perkins (89) selected two different parasite proteins, one of 155kd and another of 133kd, using Gps attached to acrylamide beads. The identification of these glycophorin binding proteins has led to the isolation of parasite genes (90) which may be useful for vaccine development.

Another experimental approach used to study the role of Gps in _P. falciparum_ invasion has been anti-Gps antibodies. Perkins (61) was the first to show that anti-Gps antibodies could inhibit invasion. She prepared a GpA-rich fraction from erythrocyte membranes, immunized rabbits with it, purified immunoglobulin G (IgG) from rabbit serum, and demonstrated that this antiserum agglutinated erythrocytes. In immunoprecipitation studies, this antiserum bound to GpA dimers, GpA monomers, and GpB, so it lacked specificity for GpA. Fab fragments of these polyclonal anti-Gps antibodies abolished the ability of soluble GpA to inhibit invasion. Merozoites pre-incubated with these Fab fragments at 1 mg/ml were also unable to invade erythrocytes. While these experiments established that anti-Gps antibodies could inhibit invasion, they did not provide any detailed information about the mechanism of invasion inhibition.

Several of the limitations of the anti-Gps antibody approach encountered with polyclonal antibodies were overcome when a series of mouse anti-Gps monoclonal

antibodies (mAb) produced by Anstee and Edwards (91) were used in invasion-inhibition experiments. The target sites of these mAb are depicted in Figure 5, and a brief description is given below.

R1.3 recognizes a common NH_2-terminal portion of GpA and GpB, and probably is targeted against saccharide moieties on them (91).

R10 is directed against the trypsin-sensitive region of GpA, which is located beteween amino acids 30 and 40 from the NH_2-terminal end (91).

R7, BRC13, BRC14, and BRC15 all react with the Wr^b antigen (91), an antigenic determinant which is located close to the membrane between amino acids 62 and 72 of GpA (73).

R18 is directed against a portion of GpA below the trypsin-sensitive region, but not the portion where the Wr^b determinant is located (91).

Pasvol et. al. (69) were the first to study the inhibitory effects of mAb R7, BRC13 and 15, and R18. When added at subagglutinating concentrations to cultures containing normal, uninfected erythrocytes and schizont-infected erythrocytes, only R7, BRC13, and BRC15 significantly inhibited invasion (54% to 100% inhibition was observed). Jungery (92) evaluated mAb R10, R18, and R7 at subagglutinating concentrations and found R7 to be the only one capable of producing strong (78%) inhibition. She also tested a variety of combinations of these antibodies and mAb R6A, which is directed against a non-glycophorin erythrocyte glycoprotein associated with the Rhesus blood

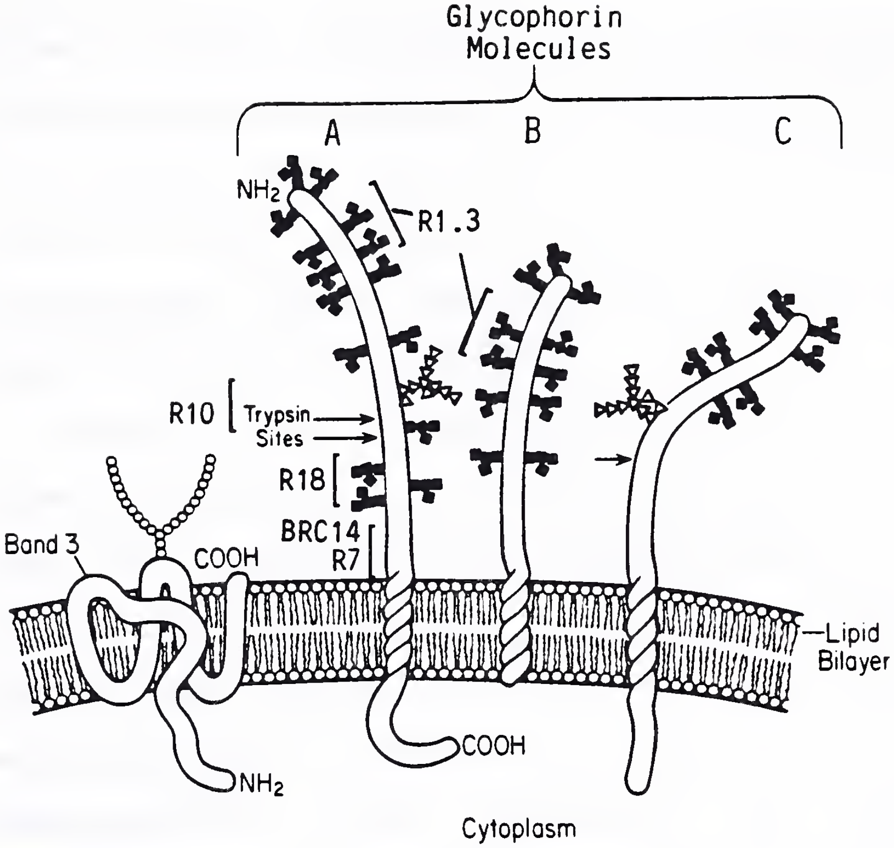


Figure 5. Target sites of anti-glycophorin A monoclonal antibodies R1.3, R7, R10, R18, and BRC14. R1.3 is directed against an epitope which is common to glycophorin A and B on their identical NH_2-terminal portions.

group antigens (Anstee, personal communication). R6A was non-inhibitory when added alone and did not appear to change the strong inhibitory effect of R7 or the non-inhibitory effect of R10 or R18 in combination with these mAb. Although R18 and R10 were not inhibitory by themselves, R10 plus R18 caused 50% inhibition.

Three other investigators have reported results of invasion-inhibition assays with these mAb (77,87,93). Facer (93), who controlled for hemagglutination by using an erythrocyte monolayer on cover slips, found R7 to be a strong (95%) inhibitor and R1.3, R6A, R10, and R18 all to be non-inhibitory. Schulman _et. al._ (77) found R7 to be non-inhibitory (<25% inhibition) at subagglutinating concentrations. R7 was, however, more inhibitory than R10 and R18 over the entire range of dilutions tested (1:20 to 1:2560). One possible explanation for Schulman's results is that he added less mAb to cultures than the other investigators, but this cannot be determined since concentration data are available only from one of the studies. Pasvol (87) found that BRC14 was the most inhibitory of a group consisting of BRC14, R1.3, R10, and R18. He observed minimal to moderate inhibition (30% to 50%) with R10 at 80 µg/ml and R18 at 70 µg/ml. Again, it is difficult to interpret these results in relation to other studies because of the lack of concentration data. Except for the result of Schulman _et. al._ (77) with R7, the results

from all of these studies suggest that a region on GpA close to the erythrocyte membrane is critical for P. falciparum invasion.

The Two-Step Model of P. falciparum Erythrocyte Invasion

The experimental results reviewed in the previous section strongly support an important role for the glycophorin family of molecules in P. falciparum invasion and suggest that two different regions of the glycophorins are involved in merozoite recognition and attachment. Pasvol et. al. (69) were the first to propose that the merozoite initially attaches to oligosaccharide molecules on glycophorins and then subsequently attaches to a site on these same molecules close to the erythrocyte membrane (the two-step model). The essential interactions encompassed in the two-step model can be summarized as follows (see Figure 6). A small group of lectin-like surface molecules on merozoites recognize and attach to specific clusters of oligosaccharides on the erythrocyte glycophorins. On GpA the O-linked tetrasaccharides attached to amino acids 10-15 and the single N-linked complex oligosaccharide appear to be more important than the tetrasaccharides on the first five NH_2-terminal amino acids. Oligosaccharides on GpB and GpC also probably contribute, but to a lesser degree than GpA. Terminal saccharide moieties such as NeuNAc and GlcNAc participate in

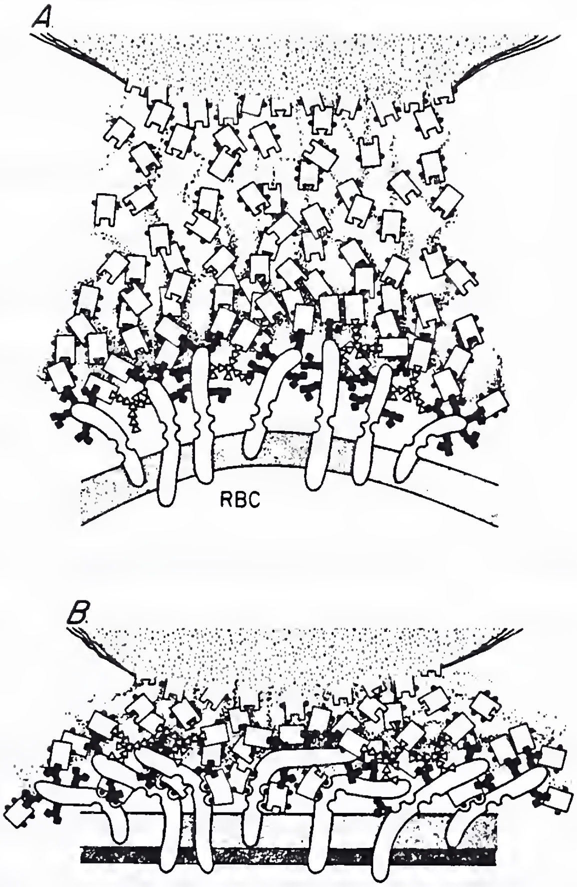


Figure 6. Proposed model of the two-step recognition and attachment process. (A) Lectin-like proteins on the merozoite bind initially to oligosaccharide on the glycophorins. (B) The merozoite makes new attachments to sites close to the erythrocyte membrane on glycophorin A and/or B and C. RBC, erythrocyte; ■, O-linked oligosaccharide; △, N-linked oligosaccharide; ⊓ and ⊓, merozoite lectin-like proteins.

binding, as do some core saccharide structures. Following this initial recognition and attachment stage, new attachments are formed to a region of the glycophorins near the membrane. This second attachment may serve to trigger the process of junction formation and erythrocyte deformation which lead to internalization of the parasite.

The experiments which contribute the best evidence for a second attachment site are invasion-inhibition studies with anti-Gps mAb. There are a number of advantages to the use of mAb in studying parasite invasion. They include : 1) mAb are targeted against specific epitopes; 2) the targets on proteins are typically less than 7 or 8 aa in size (94); 3) the potential exists to identify the parasite molecule(s) involved in invasion by making anti-idiotype antibodies to mAb. However, several potential complicating effects which make the interpretation of these results more difficult may occur when intact antibodies are added to erythrocytes. These potential complications include: 1) intact mAb are large (~150kd) relative to the glycophorins and may cause inhibition by steric hindrance; 2) bivalent antibody may cross-link Gps molecules and block binding sites or alter the deformability of the membrane (95); 3) mAb cause hemagglutination which might reduce invasion by decreasing the total surface area on erythrocytes available to merozoites.

The primary goal of the experiments described in this

thesis was to eliminate or mitigate the potential complicating effects of intact mAb by performing invasion-inhibition assays with Fab fragments of R7, R10, R18, and R6A. The possibility of invasion inhibition being produced by Gps cross-linking or hemagglutination will be eliminated with Fab fragments. Any inhibition secondary to steric hindrance should be reduced by the use of the smaller Fab fragments (~45kd versus ~150kd of intact IgG). Intact mAb were tested in this study to establish a baseline for comparison with the results of these Fab fragment experiments and studies published in the literature. All preparations of R7, R10, R18, and R6A were quantified by radial immunodiffusion to aid in comparing results between different mAb and Fab fragments. Preliminary experiments with BRC14 mAb and Fab fragments were also performed, and these results are included as well.

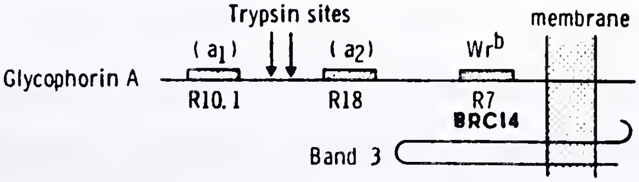
MATERIALS AND METHODS

Continous *P. falciparum* Culture

A K^+ clone of the Uganda-Palo Alto strain of *P. falciparum* was maintained in continuous *in vitro* culture as described (27, 96). The medium used was complete RPMI [RPMI 1640 (Gibco) supplemented with 3 g/L TES buffer, 2 g/L D-glucose, 2.3 g/L $NaHCO_3$, 40 mg/L gentamicin sulfate, 1.1 mg/L pyruvic acid, and 30 mg/L L-glutamine] plus 10% type A^+ or O^+ human serum. Erythrocytes were obtained from O^+Rh^+ and A^+Rh^+ donors and stored at 4°C in a 1:10 dilution of citrate phosphate dextrose for a maximum of three weeks. Cultures were incubated at 37° C in 5% O_2/5% CO_2/90% N_2. Synchronous cultures were obtained using 5% sorbitol as described (97).

Monoclonal Antibodies

The mAb used in this study were a gift of Dr. David Anstee, National Blood Transfusion Service, Bristol, England. All five mAb were raised against normal human erythrocytes and derived from mouse hybridomas. A thorough description of all except BRC14 has been published by Anstee and Edwards (91).



R6A was obtained from ascites. It is an IgG_1 mAb directed against a non-glycophorin protein on the erythrocyte membrane that may be linked to the Rhesus blood group antigens (Anstee, personal communication).

R10 was obtained from ascites. It is an IgG_1 mAb directed against the trypsin-sensitive portion of GpA.

R7 was obtained from ascites. It is an IgG_1 mAb directed against the Wr^b determinant, which appears to be located between amino acids 62 and 72 of GpA (73).

R18 was obtained from ascites. It is an IgG_{2b} mAb directed against the portion of GpA remaining after trypsin treatment, but not the Wr^b antigen.

BRC14 was obtained from hybridoma culture supernatant. It is an IgG_1 mAb and has the same anti-Wr^b specificity as R7 (98).

Fab Fragment Preparation

Purified mAb were prepared from ascites and hybridoma culture supernatant by sequential Na_2SO_4 salt fractionation at 18%, 16%, and 16% followed by extensive dialysis against 100mM PO_4, 4mM EDTA, pH 7.5 (for papain digestion) or complete RPMI (for invasion assays). Protein concentration in these preparations was calculated from A_{280} measurements. Papain digestion was performed essentially as described (99). Briefly, cysteine to a final concentration of 5mM and 2% by weight crystalline papain (Type IV, 2X crystallized, Sigma) were added to tubes containing purified mAb in PO_4-EDTA buffer. After mixing, the tubes were flushed with N_2, capped, and incubated in a 37°C water bath for 4 hours with intermittant mixing. The digestion was terminated by dialysis against several changes of distilled water over 48 hours.

The completeness of papain cleavage was assessed by immunoblotting. Samples of each digest and the corresponding intact mAb were electrophoresed under non-reducing conditions on a 3-12% gradient SDS-polyacrylamide gel using the Laemmli buffer system (100). Gel proteins were electro-blotted onto nitrocellulose paper (NCP) (Scheicher and Schuell BA85 0.2μ) as described (101). The NCP was incubated with a 1:100 dilution of peroxidase-conjugated goat anti-mouse IgG $F(ab)'_2$ antibody (Cooper Biomedical) for 2 hours. The NCP was developed using the immunoperoxidase technique as described (102). Two criteria were used to determine the extent of papain cleavage: 1) the presence of a band of 45Kd MW corresponding to Fab fragment in digest sample lanes only, and 2) the absence of a band of ~150Kd MW corresponding to intact mAb in digest sample lanes which appeared in paired intact mAb lanes.

Erythrocyte Binding Assay

The five mAb used in this study are known to bind to and agglutinate normal human erythrocytes (91 and Anstee, personal communication). Erythrocyte binding was checked prior to invasion assays to confirm that binding ability was not lost during purification and Fab fragment preparation. Dilutions of each mAb or Fab fragment ranging from 1:100 to 1:1600 were prepared in phosphate buffered saline (PBS). Identical dilutions of ascites obtained from mice injected

with SP_2 cells, a variety of myeloma cells which do not secrete immunoglobulin, were used as a control. 75 µl of each dilution was combined with 25 µl of pelleted, PBS-washed erythrocytes and the mixtures incubated at 37°C for 30 minutes with gentle mixing every 5-10 minutes. The erythrocytes were then washed with 1 ml of PBS X 3 and incubated with 75 µl of a 1:10 dilution of fluorescin-conjugated rabbit anti-mouse IgG (Cooper Biomedical) at 37°C for 30 minutes. After washing with PBS as above, thin smears were prepared and stained with Giemsa. A largefield microscope (Leitz Orthroplan) with an epifluorescent condensor was used to view slides. Each smear was assessed both for the degree of erythrocyte agglutination and fluorescence.

Radial Immunodiffusion Assays

The concentration of immunoglobulin of the IgG_1 (R6A, R7, R10) and IgG_{2b} (R18) subclasses and the concentration of Fab fragments were determined in undiluted samples using radial immunodiffusion as described (103, 104). The antisera employed were goat anti-mouse IgG_1 and IgG_{2b}, and the standards mouse IgG_1 and IgG_{2b} (all from Meloy Laboratories). To convert the values obtained from mg/ml of immunoglobulin to M/L of mAb or Fab fragment, two assumptions were made. It was assumed that most of the immunoglobulin detected was mAb or Fab fragment, and that 1.5 mg protein = $1x10^{-8}$ M protein.

Invasion Assays

The inhibitory effects of anti-glycophorin A mAb in unpurified, purified, and Fab fragment form were determined using invasion assays as described (68). Schizonts were obtained from synchronous cultures of P. falciparum using 3% gelatin (Plasmagel, Roger Bellon Laboratories, Neuilly, France)(105), and the yield of these cells determined from Giemsa-stained thin smears. Infected erythrocytes were combined with an appropriate volume of uninfected human erythrocytes so that 30 µl of this mixture provided a 1.5% hematocrit with 5% schizonts in a 200 µl culture. 30 µl of the erythrocyte mixture was added to duplicate micro tissue culture plate wells (Falcon, Micro Test III) containing 20 µl of human serum, 50 µl of complete RPMI, and 100 µl of unpurified or purified mAb diluted 1:12.5 to 1:800 in complete RPMI. Final dilutions varied from 1:25 to 1:1600. Cultures with Fab fragments also contained 30 µl of the erythrocyte mixture and 20 µl of human serum, but either 150 µl of one Fab fragment type or 75 µl of each of two Fab fragment types were added undiluted to cultures. Control cultures contained identical volumes of the erythrocyte mixture and human serum plus either intact R6A mAb or Fab fragments as above, or complete RPMI in place of mAb. All mAb and Fab fragment preparations used in invasion assays were dialyzed previously against complete RPMI. Cultures were incubated overnight at 37°C in 5% O_2/5% CO_2/90% N_2.

Thin smears of each culture were made on microscope slides, stained with Giemsa, and viewed at 1000X under oil with a light microscope (Nikon Labophot). The invasion rate was calculated as the average percentage of ring forms present in two experiments with duplicate cultures based on counts of between 1000 and 2000 cells/culture pair. Invasion as a percentage of control was calculated by comparing the invasion rate of test cultures to the invasion rate of control cultures containing no mAb. The control culture invasion rate was set at 100% to allow for comparison between different experiments.

RESULTS

The results of invasion assays performed with unpurified mAb from ascites and culture supernatant, purified mAb, and Fab fragments are given in Tables 4, 5, and 6. All of the intact mAb and Fab fragments used in invasion assays possessed the ability to bind to erythrocytes (data not shown). Both erythrocyte agglutination and a strong, generalized erythrocyte fluorescence pattern were observed at dilutions 1:100 through 1:400 of intact mAb. Only a weaker, generalized fluorescence pattern was observed at higher dilutions of intact mAb. None of the Fab fragments caused erythrocyte agglutination at any dilution tested, but all of the Fab fragments produced generalized erythrocyte fluorescence at lower dilutions. None of the Fab fragment preparations used in invasion assays contained intact mAb detectable by immunoblotting (data not shown).

Effect of Unpurified Monoclonal Antibody on Invasion

Table 4 shows that R7 produced the strongest inhibition of the four unpurified anti-glycophorin A mAb tested. The percent inhibition varied from 18.2% to 95.5% over a concentration range of 1.1×10^{-8}M to 1.7×10^{-7}M. Inhibition increased in a stepwise pattern with increasing concentration (Figure 7). R10 caused a moderate degree of inhibition ranging from 0% to 53.1% between 2.5×10^{-8}M and 4.0×10^{-7}M. The concentration of antibody in each dilution

Table 4. Results of Invasion Assays with Unpurified Monoclonal Antibodies R6A, R7, R10, R18, and BRC14 from Ascites and Culture Supernatant

Dilution	R6A[a]		R7[a]		R10[a]		R18[a]		BRC14[b]	
	Concentration[c] M/L	Percent Inhibition[d]	Concentration[c] M/L	Percent Inhibition[d]	Concentration[c] M/L	Percent Inhibition[d]	Concentration[c] M/L	Percent Inhibition[d]	Concentration[c] M/L	Percent Inhibition[d]
1:25	2.0×10^{-7}	18.0*	1.7×10^{-7}	95.5*	4.0×10^{-7}	53.1*	1.7×10^{-7}	16.5*	n.d.	29.4*
1:50	1.0×10^{-7}	15.5*	8.7×10^{-8}	87.3	2.0×10^{-7}	42.1*	8.7×10^{-8}	32.8	n.d.	28.5*
1:100	5.1×10^{-8}	7.3	4.4×10^{-8}	52.7	1.0×10^{-7}	34.1	4.4×10^{-8}	31.8	n.d.	21.3*
1:200	2.3×10^{-8}	0	2.2×10^{-8}	42.7	5.0×10^{-8}	10.9	2.2×10^{-8}	27.7	n.d.	11.9
1:400	1.3×10^{-8}	20.0	1.1×10^{-8}	18.2	2.5×10^{-8}	0	1.1×10^{-8}	0	n.d	0

[a] From ascitic fluid

[b] From hybridoma culture supernatant

[c] Calculated from radial immunodiffusion results as described in Materials and Methods

[d] Percent inhibition represents the average value of inhibition rates determined for experiments performed in duplicate. The inhibition rate is calculated as 100% (invasion rate in control cultures with no mAb set to 100%) minus the average test sample invasion rate. Average control invasion rate for experiments with R6A, R7, and R18 was 11.0% and for experiments with R10 and BRC14 was 7.7%.

n.d. Not determined

* Erythrocyte agglutination observed in cultures

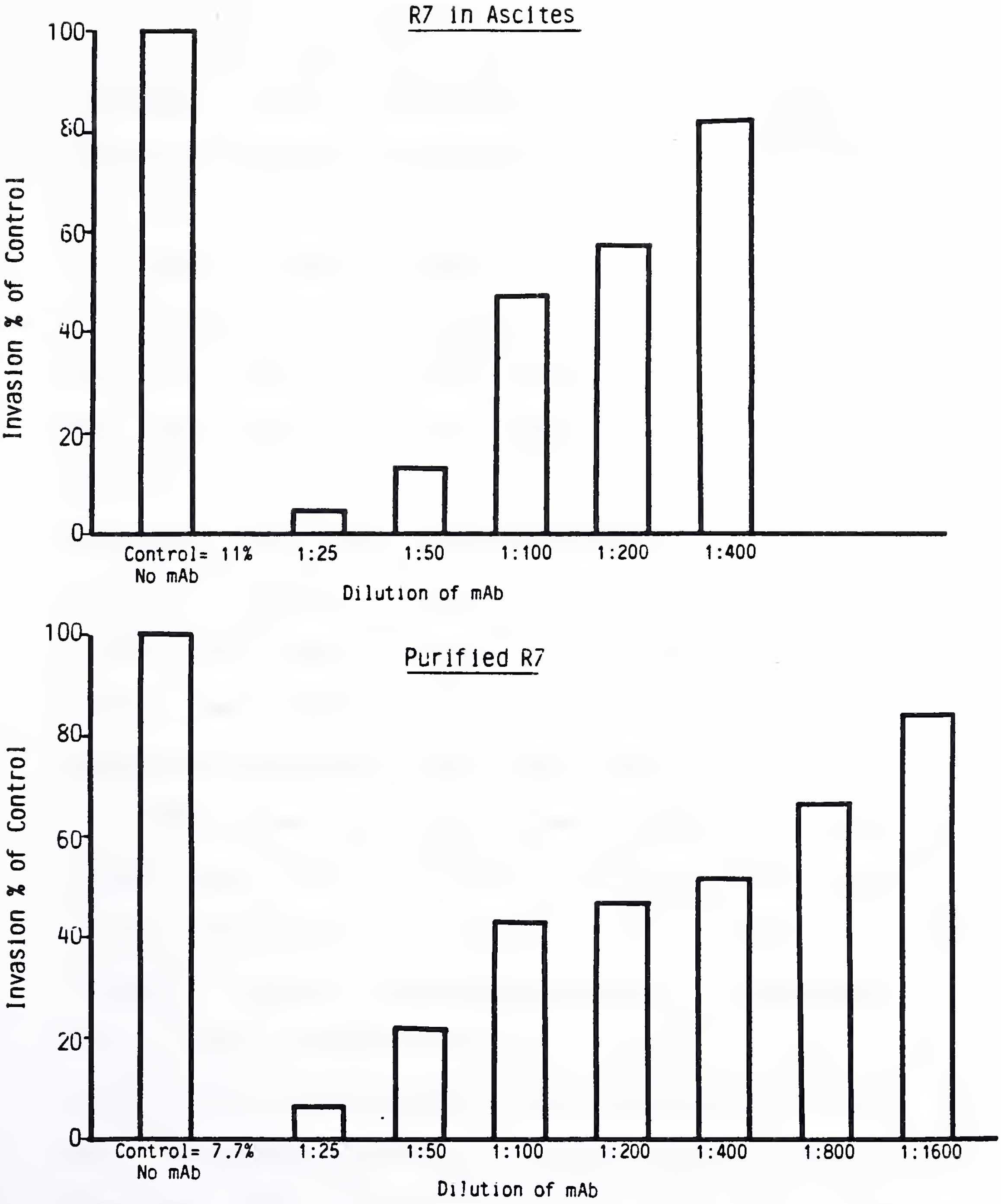


Figure 7. Inhibition of P. falciparum invasion by monoclonal antibody (mAb) R7 in ascites (top) and purified R7 (bottom) over a dilution range of 1:25 to 1:1600. Invasion rates (number of ring form parasites/100 erythrocytes in percent) in the absence of mAb were 11% and 7.7% for R7 in ascites and purified R7, respectively.

of R10 was about twice that at the same dilution of R7. Although less pronounced, an increasing pattern of inhibition similar to that observed with R7 was also noted for R10.

R18 and BRC14 both generated minimal inhibition which varied betweeen approximately 0% and 30% over the dilutions tested. R18 consistently caused less inhibition than R7 at the same concentrations. While R18 appeared to be less inhibitory than R10 over the entire dilutional range, R18 produced a similar or greater degree of inhibition at three of four comparable concentrations (compare R18 1:50 to R10 1:100, R18 1:100 to R10 1:200, and R18 1:200 to R10 1:400). Since the concentration of BRC14 was not determined, no comparison between it and other unpurified mAb can be made.

R6A caused an insignificant amount of inhibition which varied from 0% to 20% over concentrations ranging between 1.3 x 10^{-8}M to 2.0 x 10^{-7}M. This degree of inhibition occurred despite hemagglutination at dilutions 1:25 and 1:50. Less than 30% inhibition was also noted for dilution 1:25 of R18 and dilutions 1:25, 1:50, and 1:100 of BRC14 in spite of hemagglutination. Greater than 85% inhibition was observed both in the presence (R7 1:25) and absence (R7 1:50) of hemagglutination.

Effect of Purified Monoclonal Antibody on Invasion

Table 5 shows that R7 was the most inhibitory of the four purified anti-glycophorin mAb tested. R7 inhibited

Table 5. Results of Invasion Assays with Purified Monoclonal Antibodies R7, R10, R18, and BRC14

	R7		R10		R18		BRC14	
Dilution	Concentration[a] M/L	Percent Inhibition[b]	Concentration[c] M/L	Percent Inhibition[b]	Concentration[c] M/L	Percent Inhibition[b]	Concentration[c] M/L	Percent Inhibition[b]
1:25	$8.6x10^{-7}$	93.5*	$5.1x10^{-7}$	85.1*	$9.4x10^{-8}$	27.1*	n.d.	55.5*
1:50	$4.3x10^{-7}$	77.9	$2.6x10^{-7}$	71.3*	$4.7x10^{-8}$	36.2	n.d.	49.0*
1:100	$2.1x10^{-7}$	56.9	$1.3x10^{-7}$	12.7*	$2.4x10^{-8}$	31.4	n.d.	34.9*
1:200	$1.1x10^{-7}$	53.3	$6.4x10^{-8}$	15.2*	$1.2x10^{-8}$	13.6	n.d.	20.1*
1:400	$5.4x10^{-8}$	48.1	$3.2x10^{-8}$	11.4	$5.9x10^{-9}$	6.4	n.d.	29.2
1:800	$2.7x10^{-8}$	33.6	$1.6x10^{-8}$	0	---	n.d.	n.d.	6.6
1:1600	$1.3x10^{-8}$	15.6	$7.9x10^{-9}$	0	---	n.d.	n.d.	0

[a] Calculated from radial immunodiffusion results as described in Materials and Methods

[b] Percent inhibition represents the average value of inhibition rates determined for experiments performed in duplicate. The inhibition rate is calculated as 100% (invasion rate in control cultures with no mAb set to 100%) minus the average test sample invasion rate. Average control invasion rate for experiments with R6A, R7, and R18 was 11.0% and for experiments with R10 and BRC14 was 7.7%.

n.d. Not determined

* Erythrocyte agglutination observed in cultures

invasion from 15.6% to 93.5% over a concentration range of 1.3×10^{-8}M to 8.6×10^{-7}M. A pattern of greater inhibition with greater concentration was noted here as with unpurified R7 (Figure 7). Assuming that there is a linear relationship between invasion inhibition and the concentration of R7 present in culture, a surface binding curve can be plotted using least squares regression and a surface binding constant (Kd) estimated. Figure 8 shows a plot of the results for purified R7. Based upon the least squares fitted line described by the equation $Y = (1.533 \times 10^{8})X + 11.967$, the Kd of R7 can be estimated to be $\sim 1 \times 10^{-7}$M.

Unlike R10 from ascites, purified R10 produced strong inhibition to a maximum of 85.1% at 5.1×10^{-7}M. The degree of inhibition fell from 71.3% to only 12.7% with a change of dilution from 1:50 to 1:100, and all of the dilutions higher than 1:50 caused less than 15.2% inhibition. The abrupt loss of inhibitory ability did not correlate with a change in the hemagglutination pattern. At concentrations of 1.6×10^{-8}M or less, no inhibition was seen.

R18 caused essentially the same degree of inhibition in purified form as it did in unpurified form. The percent inhibition was between 6.4% and 36.2% over a concentration range of 5.9×10^{-9}M to 9.4×10^{-8}M. Compared to R10 at slightly higher concentrations, R18 appeared to be more inhibitory (compare R18 1:25 to R10 1:100, R18 1:50 to R10 1:200, and R18 1:100 to R10 1:400) although overall it again generated only minimal inhibition.

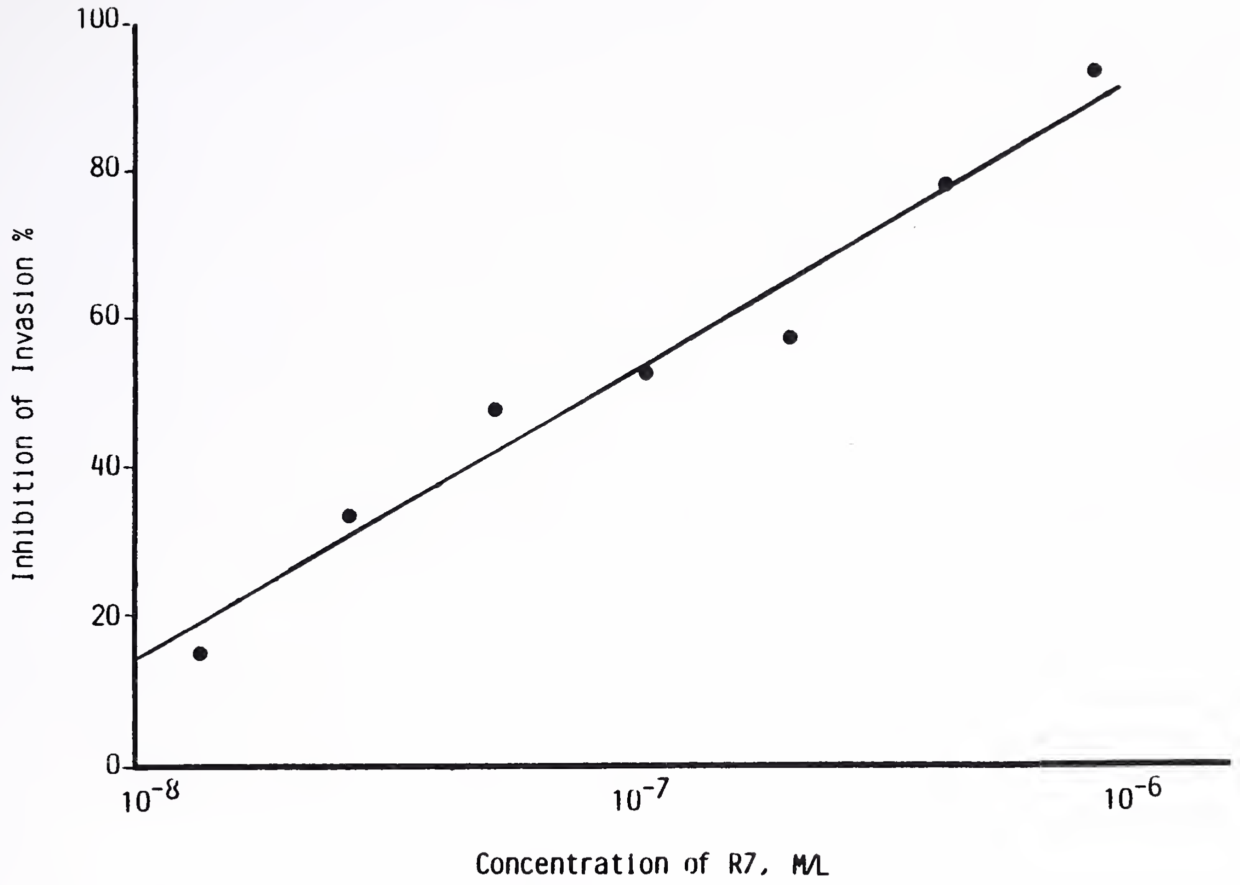


Figure 8. Surface binding curve for purified R7. The equation describing the line fitted to these data by least-squares regression is $Y = (1.533 \times 10^{8})X + 11.967$.

BRC14 was moderately inhibitory in purified form, causing between 0% and 55.5% inhibition from a 1:25 to a 1:1600 dilution. A stepwise pattern of increasing inhibition with increasing antibody concentration as observed with both preparations of R7 was also observed here. Unpurified BRC14 did not produce this pattern.

Effect of Fab Fragments on Invasion

Table 6 and Figure 9 give the results of invasion assays performed with Fab fragments used either singly or combined in pairs at concentrations 10- to 100-fold greater than those of intact mAb. R7 Fab fragments caused 87.1% inhibition at 8.5 x 10^{-6}M, which was the greatest amount of inhibition produced by any of the Fab fragments added singly. The moderate to marked inhibition observed with intact R10 was eliminated by papain cleavage, since only 10.2% inhibition was seen with R10 Fab fragments at 7.5 x 10^{-6}M. R18 Fab fragments at 13 x 10^{-6}M generated 43.4% inhibition, a value larger than any seen with intact R18. A moderate amount of inhibition, 48.3%, was observed with BRC14 Fab fragments. R6A Fab fragments produced an insignificant 5.5% inhibition at 12 x 10^{-6}M.

When the non-inhibitory R6A and R10 Fab fragments were combined at 6 x 10^{-6}M and 3.7 x 10^{-6}M, respectively, only 7.8% inhibition was noted. R18 Fab fragments at 6.5 x 10^{-6}M in combination with R6A or R10 Fab fragments generated 26.4% and 24.9% inhibition, respectively. The strong inhibitory

Table 6. Results of Invasion Assays with Fab Fragments of R6A, R7, R10, R18, and BRC14 Added Singly or in Combination

Fab Fragments	Concentration in M/L[a]	Percent Inhibition[b]
R6A	12×10^{-6}	5.5
R10	7.5×10^{-6}	10.2
R18	13×10^{-6}	43.4
R7	8.5×10^{-6}	87.1
BRC14	n.d.	48.3
R6A+R18	*	26.4
R6A+R10	*	7.8
R10+R18	*	24.9
R7+BRC14	*	66.7
R7+R18	*	90.9
R10+R7	*	97.0

[a] Calculated from radial immunodiffusion results as described in Materials and Methods

[b] Percent inhibition represents the average value of inhibition rates determined for experiments performed in duplicate. The inhibition rate is calculated as 100% (invasion rate in control cultures with no mAb set to 100%) minus the average test sample invasion rate. Average control invasion rate for experiments with R6A, R7, and R18 was 11% and for experiments with R10 and BRC14 was 7.7%.

n.d. not determined

*Concentrations of each Fab fragment used in combination experiments were exactly one half of the concentrations used for the Fab fragments added singly.

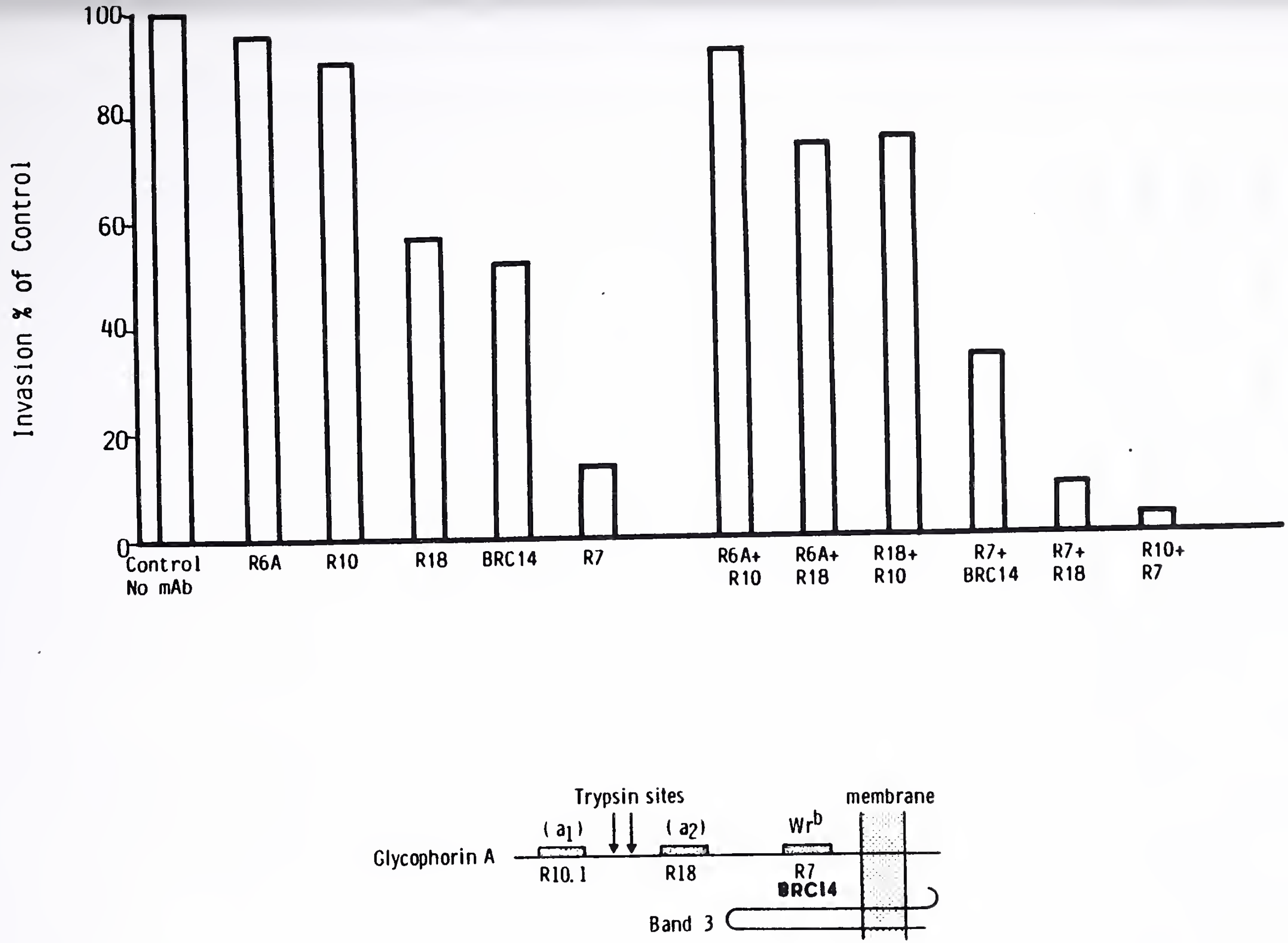


Figure 9. Inhibition of P. falciparum invasion by single or combined Fab fragments of anti-glycophorin A monoclonal antibodies. The accompanying diagram schematically represents each antibody's target site on glycophorin A.

effect of R7 Fab fragments was preserved at 4.3 x 10^{-6}M in combinations of R7+R18 and R7+R10 Fab fragments. Inhibition with the former combination was 90.9% and with the latter combination 97.0%. When R7 Fab fragments were added to BRC14 Fab fragments, which share R7's anti-Wr^b specificity, inhibition was reduced to 66.7%.

DISCUSSION

The experiments described here represent the first study of the inhibitory effects of Fab fragments of anti-GpA mAb R7, R10, R18, and BRC14 on P. falciparum invasion *in vitro*. Invasion-inhibition experiments with intact R7, R10, R18, and BRC14 were also performed to establish a basis for the comparison of results within this study and between this and previous studies.

The stong inhibitory effect of intact mAb R7, whose target site is a segment of GpA near the erythrocyte membrane, supports the contention of other investigators (69,92,93) that this region of GpA is critical for merozoite invasion. Tables 4 and 5 show that R7's greater inhibitory ability was not due to it being present in greater concentrations in test cultures. While there were no indications that R7 bound to erythrocytes better than the other mAb based on the dergee of hemagglutination or erythrocyte fluorescence in binding assays, it is possible that its surface concentration on erythrocytes was higher. R7's estimated surface binding constant of ~1 x 10^{-7}M suggests that it possesses good binding affinity for a mAb. The pattern of increasing inhibition with increasing concentrations of R7 suggests a linear relationship between invasion inhibition and mAb concentration (see Figure 7).

R7 Fab fragments at a 10-fold greater concentration than purified, intact R7 generated ~90% inhibition (Table 6), demonstrating that papain digestion did not destroy R7's

strong inhibitory ability. Both hemagglutination and direct GpA cross-linking are eliminated as potential inhibitory mechanisms by this result. If intact R7 inhibits invasion by creating steric hindrance, then R7 Fab fragments appear to retain this ability. Steric hindrance should be reduced by the use of Fab fragments, however. The fact that R7 Fab fragments combined with either R10 or R18 Fab fragments also caused strong inhibition (Figure 9) supports the idea that R7's effect dominated the effect of the other Fab fragments. However, in combination with Fab fragments of BRC14, which share R7's anti-Wr^b specificity and attach to a similar site on GpA, inhibition was reduced (Figure 9). Since BRC14 Fab fragments appeared to be less inhibitory than R7 Fab fragments, this reduction might be the result of BRC14 fragments binding to GpA and lowering the density of the more inhibitory R7 fragments.

The minimal degree of inhibition (peak inhibition of 35%, Tables 4 and 5) observed with intact mAb R18 suggests that it does not bind to a site on GpA which is critical for merozoite invasion. While intact, purified R18 did not cause strong inhibition at the same concentrations as R7 (Table 5), there are indications that a concentration effect occurs with R18. As noted in the RESULTS section, intact R18 appeared to be at least as inhibitory as the moderately inhibitory R10 at similar concentrations. In addition, R18 Fab fragments produced ~50% inhibition at concentrations approximately 75- to 150-fold greater than the peak

concentrations of intact R18 tested. Finally, when R18 Fab fragments were combined at 6.5 x 10^{-6}M (i.e. one half the concentration of R18 fragments added singly) with the non-inhibitory R10 or R6A fragments, inhibition was reduced (Figure 9). If greater concentrations of intact R18 had been added to cultures, a moderate degree of inhibition like that caused by R10 might have occurred. A concentration effect may explain the different degrees of inhibition reported for R18 in the literature (69,77,87,92,93). The target site for R18 on GpA is more distal than but still close to the target site for R7. The binding of intact R18 or higher concentrations of R18 Fab fragments may partially block merozoites from binding to attachment sites closer to the erythrocyte membrane.

The moderate to strong inhibition observed at peak concentrations of intact R10 was not seen with Fab fragments at 7.5 x 10^{-6}M (Figure 9), suggesting that bivalent R10 molecules inhibit by a mechanism such as GpA cross-linking or steric hindrance. Because R10 binds to GpA in a region which is close to the oligosaccharides, it may hinder the initial attachment of merozoites to erythrocytes. R10 Fab fragments in combination with R6A or R7 Fab fragments did not alter the degree of inhibition seen when the latter fragments were added singly, suggesting that R10 was also non-inhibitory in these cultures. The relationship between percent inhibition and concentration of intact R10 does not appear to be linear (Table 5), a finding which suggests the

possiblity of interactions other than the simple attachment of R10 to its GpA target site (e.g. at higher concentrations, R10 might bind to itself and aggregate clusters of GpA molecules).

Monoclonal antibody BRC14 was obtained from Dr. David Anstee near the end of these experiments, so this mAb was not as thoroughly studied. Although BRC14 appeared to be only minimally inhibitory when added in unpurified form (Table 4), it produced a moderate degree of inhibition in purified form (Table 5) and retained its inhibitory ability in Fab fragment form. BRC14 would be expected to inhibit invasion strongly based on its target region similarity with R7. BRC14's lesser inhibitory ability could be due to a lower binding affinity, lower concentrations in culture, or a target region slightly removed from the merozoite attachment site. The difficulties encountered in interpreting the BRC14 results emphasize the importance of quantifying the amount of antibody used in invasion-inhibition experiments.

Hemagglutination has been suggested as a possible inhibitory mechanism by two investigators (87,93). The control mAb in this study, R6A, produced insignificant inhibition despite causing hemagglutination at higher concentrations (Table 4). This observation demonstrates that hemagglutination alone does not necessarily cause inhibition. Since essentially normal invasion rates were observed in the presence of R6A (Table 4), inhibition

also does not necessarily occur because the erythrocyte surface is covered with antibody. R6A did not produce significant inhibition in any of the studies where it was tested (77,87,92,93).

Although these anti-GpA mAb studies are subject to fewer interpretational difficulties than previous studies using unquantified amounts of intact mAb, they nonetheless have several limitations. The chief constraint on this study was the limited quantity of mAb available. All of the mAb were a gift of Dr. David Anstee, and the original amount of each ascites or culture supernatant sample was 3.0 ml to 4.5 ml. There was an insufficient quantity of mAb to perform experiments more than two times after preliminary studies on antibody binding, papain digestion conditions, and immunoglobulin purification methods were done. Since the mean values of the results of experiments with different antibody and Fab fragment preparations are represented by an N equalling 2, an analysis of statistical differences was not performed.

In addition to the limitations imposed by the small amount of mAb available, time limitations did not allow me to perform two other control experiments. Although unlikely, some fraction of the inhibition observed with mAb could have been caused by toxic effects on intracellular parasite development. No reports of experiments to control for this possiblity appear in the literature. Toxic effects could be ruled out by adding varying concentrations of mAb

and Fab fragments to synchronized cultures containing ring form parasites and monitoring development over a 40-48 hour period. Secondly, a control to eliminate the possibility that these mouse mAb bind to human immunoglobulins attached to the erythrocyte surface (e.g. the mAb might have anti-rheumatoid activity) would also have been useful. This experiment could be performed by attaching immunoglobulins from human serum to the inside of wells in a plastic dish, adding the mAb and Fab fragments to the wells, and determining if binding occurs.

The surface binding curve shown in Figure 8 was drawn on the assumption of a linear relationship between percent inhibition and concentration of mAb. This assumption would be incorrect if bound mAb caused interactions between GpA molecules which led to phenomena such as aggregation. The assumption of a linear relationship between percent inhibition and antibody concentration does not depend on direct blockage of a merozoite binding site nor does it depend on a linear correlation between mAb binding and concentration.

An important limitation of this and all other in vitro inhibition studies is that the intricacies of invasion occur in what largely remains a "black box". The light and electron microscope studies reviewed in the third section have revealed merozoite invasion to be a complex, multi-step process (7-11, 13). Invasion assays performed with synchronized, schizont-infected erythrocytes provide us only

with invasion rates at the endpoint of the process. While these rates suggest what interactions are taking place between the disappearance of schizonts and the appearance of ring forms, they provide no direct proof of mechanism. For example, inhibition produced by the addition of exogenous saccharides can be caused by inhibitory effects on parasite development as well as merozoite invasion (86). The creation of invasion assay systems using viable, isolated merozoites (62,107,108) represents the initial step in attempts to separate clearly inhibitory and toxic effects.

Inhibitory mechanisms other than GpA cross-linking, hemagglutination, and steric hindrance may complicate the interpretation of invasion-inhibition experiments with mAb. Erythrocyte deformability appears to play a significant role in *P. falciparum* invasion (109-111). Chasis *et. al.* (95) have shown that R10 and its Fab fragment may decrease membrane deformability by inducing an association between GpA and the cytoskeletal network. The results with R10 Fab fragments in this study do not suggest that a change in deformability affecting invasion occurred, but R7 could conceivably inhibit by this mechanism. The importance of hydrophobic interactions between merozoites and erythrocytes has also been demonstrated (58,62,64,86). By binding close to the membrane and to molecules of high density, R7 may prevent merozoites from attaching to the hydrophobic segments of other erythrocyte proteins important for invasion. The dramatic vacuolization which follows junction

formation must involve intimate contact between merozoite and erythrocyte membranes. After initial attachment occurs, intermembranous hydrophobic interactions could lead to junction formation and the inititation of parasite entry.

Although many of the details of the _P. falciparum_ invasion process are unknown, the identification of glycophorins as erythrocyte receptors has already led to progress in the search for a vaccine against merozoites. For an anti-malarial vaccine to be effective, it must inhibit some process critical for parasite survival and be targeted at molecules that vary minimally or not at all between members of a given species. Recognition of and attachment to erythrocytes are essential to parasite survival, since this activity allows for successive intra-erythrocytic cycles of parasite development. Also, it would be expected that the merozoite proteins responsible for binding to a specific erythrocyte molecule would not be highly variable.

As mentioned earlier, Perkins (89) found two late-stage parasite proteins of 155kd and 133kd MW which appeared to have high affinity for Gps and represent glycophorin binding proteins (GBP). Antibodies to both effectively inhibited invasion, suggesting that these proteins did have an essential role in the erythrocyte-merozoite interaction. Based on these encouraging results, Ravetch _et._ _al._ (90) searched a cDNA library of late-schizont DNA for sequences whose expressed proteins reacted with antiserum raised

against the 155kd and 133kd proteins. Several clones were identified, and all shared a 150 nucleotide tandem-repeating sequence coding for a 50 amino acid repeat. The epitope represented by these repeat sequences thus appeared to be the target of the inhibitory antibodies against the GBP.

The discovery of antibody-inducing epitopes comprised of tandemly repeated amino acid sequences on the surface proteins of merozoites [and also sporozoites (112)] should hasten the development of stage-specific vaccines consisting of these repeat proteins. A merozoite vaccine of this type would be expected to lead to immune responses capable of directly interfering with merozoite invasion in an infected host.

During the discussion of the advantages of using mAb to study invasion mechanisms, it was pointed out that mAb can be utilized to generate anti-idiotype antibodies which might aid in the identification of merozoite proteins involved in erythrocyte invasion. Because R7 proved to be a strong inhibitor both as an intact antibody and as Fab fragments in this study, work is now in progress in the laboratory of Dr. Michele Jungery to raise and characterize anti-idiotype antibodies to R7. The results of these studies would be of potential usefulness for the development of stage-specific anti-merozoite vaccines and would contribute to a better understanding of the details of the invasion process.

As promising as stage-specific vaccines appear on paper

and in controlled experiments, it should be remembered that the gap between an agent's efficacy in the laboratory and its effectiveness in the field may be enormous. In order to succeed in controlling an organism as adaptive as Plasmodium, an equal effort will have to be applied to the details of vaccine deployment. In many of the developing countries where a reduction in malaria morbidity and mortality would significantly improve the health of the population, no basic health care infrastructure now exists to implement an organized vaccination campaign. For this and other economic, social, and cultural reasons, malaria may continue to be a parasitic disease of worldwide importance even in the upcoming age of malaria vaccines.

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